IMMUNOBIOLOGY

# Characterization of human blood dendritic cell subsets

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**Dendritic cells (DCs) are key antigen-presenting cells for stimulating immune responses and they are now being investigated in clinical settings. Although defined as lineage-negative ($Lin^-$) $HLA\text{-}DR^+$ cells, significant heterogeneity in these preparations is apparent, particularly in regard to the inclusion or exclusion of $CD14^+$, $CD16^+$, and $CD2^+$ cells. This study used flow cytometry and a panel of monoclonal antibodies (mAbs), including reagents from the 7th Leukocyte Differentiation Antigen Workshop, to define the cellular composition of 2 standardized peripheral blood mononuclear cell (PBMCs)–derived $Lin^-$ $HLA\text{-}DR^+$ preparations. $Lin^-$ cells were prepared from PBMCs by depletion with CD3, CD14, CD19, CD11b, and either CD16 or CD56 mAbs. Analysis of the CD16-replete preparations divided the $Lin^-$ $HLA\text{-}DR^+$ population into 5 nonoverlapping subsets (mean ± 1 SD): CD123 (mean = 18.3% ± 9.7%), CD1b/c (18.6% ± 7.6%), CD16 (49.8% ± 8.5%), BDCA-3 (2.7% ± 1.4%), and CD34 (5.0% ± 2.4%). The 5 subsets had distinct phenotypes when compared with each other, monocytes, and monocyte-derived DCs (MoDCs). The CD85 family, C-type lectins, costimulatory molecules, and differentiation/activation molecules were also expressed differentially on the 5 $Lin^-$ $HLA\text{-}DR^+$ subsets, monocytes, and MoDCs. The poor viability of $CD123^+$ DCs in vitro was confirmed, but the $CD16^+$ $CD11c^+$ DC subset also survived poorly. Finally, the individual subsets used as stimulators in allogeneic mixed leukocyte reactions were ranked by their allostimulatory capacity as CD1b/c > CD16 > BDCA-3 > CD123 > CD34. These data provide an opportunity to standardize the DC populations used for future molecular, functional and possibly even therapeutic studies. (Blood. 2002; 100:4512-4520)**



## Introduction

Dendritic cells (DCs) are specialist antigen-presenting cells that originate from the bone marrow and play critical roles in the initiation and direction of immune responses.[1,2] They are being investigated in cancer biology, transplantation, and autoimmunity. The definition of a DC, to date, has been mainly a functional one.[2] The ability of DCs to take up, process, and present antigens to stimulate T (and B) lymphocytes is accompanied by certain, less consistent, phenotypic and morphologic features. DCs lack certain lineage (Lin)–specific markers and express high levels of major histocompatibility complex (MHC) class II molecules; thus, the phenotypic definition of DC as $Lin^-HLA\text{-}DR^+$ cells has become widespread. Their relatively low frequency in leukocyte preparations[3,4] and, in particular, the paucity of DC-specific reagents, has hampered their investigation. This has meant that purification of DCs has and continues to depend heavily on their $Lin^-$ status. However, recent evidence suggests that one or more DC or DC-like populations may express CD14, CD2, or CD16, which have traditionally been associated with $Lin^+$ cells.[5-7] Interlaboratory variation in the particular combination of Lin monoclonal antibody (mAb) and purification methodologies (rosetting, magnetic bead depletion, flow cytometric sorting) used, is likely to result in DC preparations with different compositions.

Human blood DC preparations contain several phenotypically and functionally distinct subpopulations.[8,9] Their constitution, function, and lineage of origin still require clarification. The original "myeloid" $CD11c^+CD123^{lo}$ DC subset is now contrasted with the $CD11c^-CD123^{hi}$ "lymphoid" DC population.[10] Other markers including CD33, CD16, CD2, CD1, and CD85 (immunoglobulinlike transcript; ILT)[11] have been used to further fractionate these populations. Thus, CD33 density has been suggested to define a mature and immature myeloid subset[6] and recent reports of a $CD16^+$ DC population[5,12] may extend these to include the previously described $CD16^+CD14^{lo}$ monocyte population.[13,14] CD2 has been described on a subset of $CD14^+$ leukocytes, which exhibit DC characteristics including the capacity for priming naive T lymphocytes.[7] CD1a was reported to demarcate a $Lin^-$ population, which acquired Langerhans cell (LC) features in vitro[15]; however, this population has been reinvestigated and redefined as a $CD1a^-CD1b/c^+$ population.[16] The CMRF-44 mAb appeared to define 3 blood DC populations after a brief period of in vitro culture.[17] BDCA-3 mAb identifies another subpopulation.[18]

Knowledge of the relative contributions of the DC subsets to a defined $Lin^-HLA\text{-}DR^+$ preparation, would be useful. So, too, would a direct comparison of the phenotypic and functional properties of subsets. This might assist attempts to define whether these apparent subsets represent different stages of differentiation/activation of the same lineage or the progression of DC differentiation pathways. In this study, we examined the heterogeneity of human peripheral blood mononuclear cell (PBMC)–derived DC preparations using a wide panel of mAbs, many of which were made available by colleagues participating in the DC section of the 7th Leukocyte Differentiation Antigen Workshop (LDAW).[19] Emphasis was placed on identifying subsets with restricted expression

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Submitted November 30, 2001; accepted June 24, 2002. Prepublished online as *Blood* First Edition Paper, August 15, 2002; DOI 10.1182/blood-2001-11-0097.

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of molecules with known functions, which might therefore delineate functionally distinct subsets.

## Materials and methods

Monoclonal and polyclonal antibodies used in the study are listed in Table 1.

### Cell purification

Buffy coats from healthy donors were obtained from the Australian Red Cross Service (Brisbane, Australia). PBMCs were isolated by standard density gradient centrifugation over Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden). PBMCs were stained with mAb cocktails designed either to include CD16$^-$ cells (CD3 [OKT3], CD14 [CMRF-31], CD19 [FMC-63], CD11b [OKM1], CD56 [NKH-1]) or to exclude them (CD3, CD14, CD19, CD11b, CD16 [HuNK-2]). CD11b was included to ensure monocyte-negative selection was adequate and because several investigations have shown all blood DCs lack CD11b.[6,20] Following washing, the cells were incubated with Biomag goat anti–mouse-immunoglobulin–coated magnetic beads (Polysciences, Warrington, PA). Labeled cells were depleted by first preclearing with a MCP-1 magnet (Dynal, Oslo, Norway) followed by passing through a magnetic-activated cell sorting (MACS) CS column on a Variomacs magnet (Miltenyi Biotech, Gladbach, Germany). For some experiments, Lin$^-$HLA-DR$^+$ cells were sort purified (FACS Vantage, Becton Dickinson, San Jose, CA) using 2-color labeling with phycoerythrin (PE)–conjugated CD3, CD56, and CD20 to gate out contaminating Lin$^+$ cells in combination with HLA-DR-PE-cyanin 5.1 (Cy5). For cell activation and functional studies Lin$^-$ preparations were labeled with fluorescein isothiocyanate (FITC)–conjugated lineage mAb (CD56, CD3, CD14, and CD20) and various combinations of PE-conjugated or PE-Cy5–conjugated subset mAbs (CD123, CD16, CD1b/c, BDCA-3, and CD34), and the desired Lin$^-$ subsets sorted.

For T-cell purification, PBMCs were fractionated by incubation with neuraminidase-treated sheep red blood cells followed by separation of the rosetting (ER$^+$) and nonrosetting (ER$^-$) populations on Ficoll density gradients. After lysis of the ER$^+$ fraction with 0.15M $NH_4Cl$, pure populations of responder T cells were prepared by magnetic immunodepletion with CD14 (CMRF-31), CD19 (FMC-63), CD16 (HuNK-2), CD11b (OKM1), and HLA-DR (L243) mAbs. The resulting cells were 96% to 100% CD3$^+$.

For generation of monocyte-derived DCs (MoDCs), the nonrosetting population was cultured at $0.3 \times 10^6$ CD14$^-$ monocytes/mL in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 200 U/mL, Sandoz-Pharma, Basel, Switzerland) and interleukin 4 (IL-4; 50 U/mL, Sigma, St Louis, MO) for 5 days.[21]

### Cell staining

Cells were incubated with mAb according to the manufacturer's instructions, or at 5 to 20 μg/mL in 2% fetal calf serum (FCS) in phosphate-buffered saline (PBS), or in hybridoma culture supernatant for 20 to 60 minutes at 4°C. Cells were washed in 2% FCS in PBS. Unconjugated mAbs were detected with the appropriate conjugated secondary detection reagents diluted in 2% FCS in PBS. For phenotypic analysis, following staining the cells were washed and fixed with 1% paraformaldehyde prior to analysis on a FACS Calibur cytometer (Becton Dickinson). For cell survival studies, unfixed cells were analyzed, and propidium iodide (PI; 3 μg/mL) was used to identify dead cells. Cytoplasmic staining, using "Fix & Perm" (Caltag, Burlingame, CA), for langerin was done on PBMCs by 4-color flow cytometry using biotinylated antilangerin mAb (CD207, DCGM-4) detected with streptavidin-peridinin chlorophyll protein (PerCp). DCs were identified as surface-labeled Lin mAb-FITC$^-$/HLA-DR-APC$^+$ events, and DC subsets were identified with the appropriate PE conjugates.

### DC culture

Sort-purified Lin$^-$CD11c$^+$ (± CD16$^+$ cells) or Lin$^-$CD11c$^-$ cells were incubated overnight at $10^6$ cells/mL in RPMI 1640 with 10% FCS,

**Table 1. mAb and polyclonal reagents used in this study**

| CD/name | Name/clone | Conjugate | Source |
|---|---|---|---|
| Lineage-depletion mAb | | | |
| CD3 | OKT3 | Nil | ATCC[a] |
| CD11b | OKM1 | Nil | ATCC |
| CD14 | CMRF31 | Nil | In house |
| CD16 | HuNK-2 | Nil | I. McKenzie[b] |
| CD19 | FMC63 | Nil | H. Zola[c] |
| CD56 | NKH-1 | Nil | Beckman Coulter[d] |
| HLA-DR | L243 | Nil | ATCC |
| Phenotyping mAb | | | |
| BDCA-3 | — | PE, FITC | Miltenyi |
| CD1b/c[e] | M306 | Biotin | Immunex[e] |
| CD1c[e] | BDCA-1 | FITC, PE | Miltenyi |
| CD2 | T11 | PE, FITC | Coulter |
| CD4 | Leu-3a | PE | BD |
| CD7 | M-T107 | PE | BD |
| CD11c | Leu-M5 | PE | BD |
| CD11c | 3.9 | FITC | Southern Biotech[f] |
| CD14 | Leu-M3 | PE | BD |
| CD16 | Leu-11a/c | PE, FITC | BD |
| CD20 | B9E9 | PE | Immunotech[g] |
| CD33 | P67.6 | PE | BD |
| CD34 | Anti-HPCA-2 | PE, FITC | BD |
| CD40 | MAB 89 | PE | Immunotech |
| CD52 | YTH34.5 | FITC | Serotec[h] |
| CD56 | NKH-1 | PE | Immunotech |
| CD64 | 10.1 | PE | Serotec |
| CD64 | 10.1 | FITC | Pharmingen[i] |
| CD80/B7-1 | BB1 | PE | Pharmingen |
| CD83 | HB15a | Nil, FITC, PE | Immunotech |
| CD85a/ILT-5 | 7H5 | Nil | A van Agthoven[j] |
| CD85d/ILT-4 | 42D1 | Nil | A van Agthoven |
| CD85j/ILT-2 | HP-F1 | Nil | A van Agthoven |
| CD85j/ILT-2 | VMP55 | FITC | Dako[k] |
| CD85k/ILT-3 | ZM3.8 | Nil | A van Agthoven |
| CD86/B7-2 | 2331 | PE | Pharmingen |
| CD123 | 7G3 | PE | Pharmingen |
| CLA | HECA-452 | FITC | Pharmingen |
| CMRF-44 | — | Biotin | In house |
| CMRF-56 | — | Biotin | In house |
| DEC-205/CD205 | MMRI-7 | Biotin | In house |
| DEC-205/CD205 | M335 | Biotin | Immunex[l] |
| DC-SIGN/CD209 | AZN-D1 | Nil | Y. van Kooyk[l] |
| HLA-DR | Immu-35T | PC5 | Immunotech |
| HLA-DR | B-F1 | FITC | Serotec |
| Langerin/CD207 | DCGM-4 | Nil | Schering-Plough[l] |
| MMR/CD206 | MMR 190.BB3 | Nil | F. Sallusto[l] |
| Detecting reagents | | | |
| Anti-mouse Ig | Polyclonal | FITC, PE | Silenus[l] |
| Anti-mouse Ig | Polyclonal | Biotin | Sigma[m] |
| Streptavidin | — | PE, PE-Cy5 | Dako |
| Avidin | — | FITC | BD |

ATCC indicates American Type Culture Collection; BD, Becton Dickinson.

[e]CD1b/c (M306) and CD1c (BDCA-1) used interchangeably as required. Denoted as CD1b/c in text and in figures.

[a]Manassas, VA

[b]Austin Research Institute, Melbourne, Australia

[c]Child Health Research Institute, Adelaide, Australia

[d]Gladesville, Australia

[e]Seattle, WA

[f]Birmingham, AL

[g]Beckman Coulter, Gladesville, Australia

[h]Raleigh, NC

[i]BD Biosciences, San Diego, CA

[j]7th LDAW c/o Nuffield Department of Clinical Biochemistry and Cellular Science

[k]Botany, NSW, Australia

[l]Chemicon, Boronia, Victoria, Australia

[m]Sydney, Australia

10 ng/mL IL-3 (Gibco BRL, Grand Island, NY) and 200 U/mL GM-CSF, or were cocultured in the absence of cytokines with allogeneic $CD3^+$ T cells at a 1:1 ratio.

### Allogeneic MLRs

Allogeneic mixed leukocyte reactions (MLRs) were established using various numbers of each $Lin^-$ subset cultured in triplicate in round-bottom 96-well tissue culture plates (Costar, Acton, MA) with $10^5$ freshly isolated allogeneic T cells, at 37°C in 5% $CO_2$ for 5 days. T-cell proliferation was measured by the uptake of [$^3$H]-thymidine (1 μCi/well [0.037 MBq/well]; 6.7 Ci/mM [248 MBq/mM]; Amersham, Buckinghamshire, United Kingdom), which was added 18 hours prior to harvesting. Cells were harvested onto glass fiber filter paper with an automated harvester (TomTec Mach III, Hamden, CT) and [$^3$H]-thymidine incorporation was measured by liquid scintillation spectroscopy (Wallac, Turku, Finland). Responses are reported as mean cpm ± SEM for triplicate wells.

## Results

### Analysis of the composition of $Lin^-HLA\text{-}DR^+$ PBMC preparations

We prepared 2 types of $Lin^-$ PBMC preparations using either CD16 or CD56 mAb to deplete natural killer (NK) cells, the latter with a view to studying the $CD16^+$ DC population.[5,12] The resulting CD16- and CD56-depleted $Lin^-$ preparations were examined for purity, assessed by reactivity with FITC-conjugated sheep anti-mouse immunoglobulin (FITC-SAM) to detect contaminating $Lin^+$ cells. The majority (75% ± 25.8% SD, n = 14) of cells within both types of $Lin^-$ preparations were $Lin^-$ (R1 in Figure 1) or weakly fluorescent (within the second decade of fluorescence intensity, R2 in Figure 1). The remaining strongly labeled cells were considered to be contaminating $Lin^+$ cells (R3, Figure 1A,F).

The composition of the $Lin^-$ preparations was further assessed by 3-color flow cytometry using FITC-SAM, PE-Cy5–HLA-DR and a panel of PE-conjugated lineage markers. Both CD16- and CD56-depleted $Lin^-$ preparations contained an exclusively $HLA\text{-}DR^+$ population within R1 and R2, whereas the cells within R3 were largely $HLA\text{-}DR^-$ (Figure 1B,G). In CD16-depleted preparations, the cells within R2 expressed HLA-DR at a higher density than those in R1. This difference was barely evident in CD56-depleted preparations. Gating on the $Lin^-$ $HLA\text{-}DR^+$ cells (R4, Figure 1B,G) demonstrated that cells from both types of preparation lacked $CD20^+$ B cells (Figure 1C,H), but a small population of $CD7^+$ cells was consistently observed in R1 (Figure 1D,I). In both preparations, cells within R2 expressed low levels of CD64, with a modest correlation between the intensity of CD64 and low-density $Lin^+$ marker staining (Figure 1E,J). No $CD64^+$ cells were found within R1. Variable CD14 expression within both preparations contributed to the low-intensity $Lin^+$ labeling in R2 and correlated with $Lin^+$ intensity (Figure 1K,P). The CD56-depleted preparations contained increased numbers of $CD14^+$ cells. As expected, $CD16^+$ cells were present in the CD56-depleted $Lin^-$ cell populations (Figure 1 L,Q). A disperse $HLA\text{-}DR^+CD56^+$ population was detected in the $Lin^-CD16$-depleted preparations with residual positive cells also found in the CD56-depleted preparations. These were largely restricted to R2 (Figure 1M,R).

As expected, $Lin^-HLA\text{-}DR^+$ cells were divided into substantial $CD11c^-$ and $CD11c^+$ populations, with the 2 populations localized to R1 and R2, respectively (Figure 1N,S). Staining with CD33 divided CD16-depleted $Lin^-$ preparations into 2 subsets (Figure 1O). In contrast, CD56-depleted $Lin^-$ preparations contained 3 cell populations, based on CD33 expression (Figure 1T). Thus, whereas all myeloid $Lin^-$ cells uniformly expressed CD11c, the $Lin^-HLA\text{-}DR^+CD16^+$ cells could be distinguished by their low-density CD33 expression from their $HLA\text{-}DR^+CD11c^+CD16^-$ counterparts. $Lin^-CD33^{lo}$ cells have been described as immature blood DCs.[22] Subsequent $Lin^-$ subset analyses were performed on CD16-containing, CD56-depleted $Lin^-$ preparations.

### CD123, CD1b/c, CD16, BDCA-3, and CD34 subdivide the $Lin^-HLA\text{-}DR^+$ preparations into 5 distinct nonoverlapping subsets

CD16,[5,12] CD1c,[15,18,16] and BDCA-3[18] have recently been identified as markers that subdivide the $CD11c^+$ DC population. $CD123^-$, $CD123^{lo}$, and $CD123^{hi}$ subpopulations are present in $Lin^-$ preparations.[9] Furthermore, $CD34^+$ cells have been documented as components of $Lin^-HLA\text{-}DR^+$ preparations.[20] We, therefore, examined the relative frequency and the level of expression of HLA-DR and CD11c by each of the 5 $Lin^-$ subsets defined using these reagents (CD16, CD1b/c, BDCA-3, CD123, and CD34).

Initial 2-color analysis confirmed that each of the 5 subsets was present in the $Lin^-$ preparations. Whereas the $CD16^+$ and $CD1b/c^+$ subsets were largely restricted to R2, the $CD123^+$ and $CD34^+$ subsets were localized within R1 (Figure 1). In contrast, the BDCA-3 subset was dispersed between both regions (Figure 2A-E). The relative expression of HLA-DR and CD11c by the 5 populations was next examined on sort-purified $Lin^-HLA\text{-}DR^+$ cells by 3-color staining. The $CD16^+$ cells were identified as $CD11c^{hi}$ cells (Figure 2K), which exhibit a lesser overall density of HLA-DR (Figure 2F). Labeling with CD1b/c identified an $HLA\text{-}DR^{hi}$ subpopulation (Figure 2G), which also expressed the highest levels of CD11c (Figure 2L; note that different fluorochrome conjugates of CD11c were used in Figure 2K-O). Notably, the highest levels of CD1b/c expression directly correlated with the highest levels of both HLA-DR and CD11c. BDCA-$3^+$ DCs expressed CD11c and moderate to high levels of HLA-DR (Figure 2H,M). The $CD123^{hi}$ subset had moderate- to high-density HLA-DR expression but was $CD11c^-$ (Figure 2I,N). The $CD34^+$ subset was $CD11c^-$ and expressed only low levels of HLA-DR (Figure 2J,O). Thus, the expression of CD16, BDCA-3, and CD1b/c was restricted to $CD11c^+$ cells and CD123 and CD34 to $CD11c^-$ cells. These 5 $Lin^-$ populations expressed differential levels of HLA-DR.

Further 3-color analysis of a series of CD56-depleted $Lin^-$ preparations stained with HLA-DR, CD11c, and one of CD1b/c,

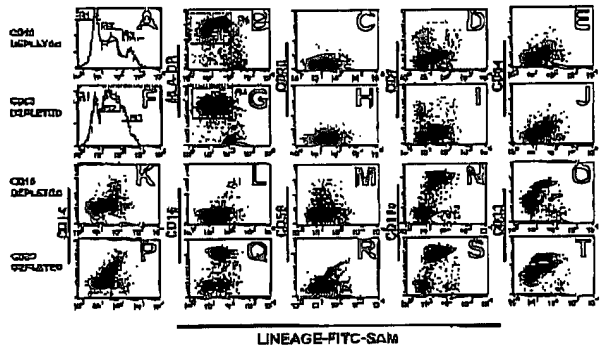


**Figure 1. Human $Lin^-$ PBMCs.** Analysis of human $Lin^-$ PBMC preparations obtained using a mAb mix containing either CD16 (A-E and K-O) or CD56 mAb (F-J and P-T). $Lin^-$ cells were stained with FITC-SAM (to detect residual $Lin^+$ cells) and HLA-DR in conjunction with one of a panel of lineage mAbs. Flow cytometry profiles of (A) CD16-depleted or (F) CD56-depleted residual lineage-labeling intensity shows 3 peaks: R1, R2, and R3. The $Lin^-HLA\text{-}DR^+$ cells (B,G) were analyzed further for CD20 (C,H), CD7 (D,I), CD64 (E,J), CD14 (K,P), CD16 (L,Q), CD56 (M,R), CD11c (N,S), and CD33 (O,T).

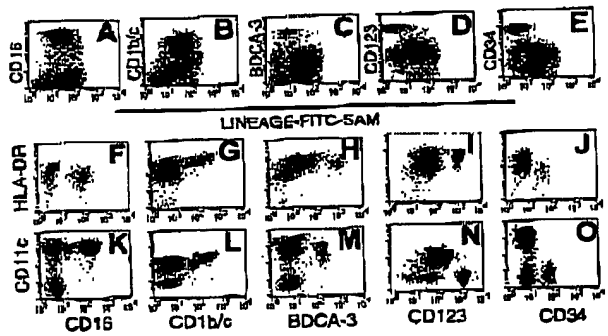


**Figure 2. Phenotypic analysis of Lin⁻ PBMC preparations identifies 5 phenotypic subsets.** CD56-depleted Lin⁻ preparations were stained with FITC-SAM and either CD16, CD1b/c, BDCA-3, CD123, or CD34. Live cells were gated based on forward- and side-scatter characteristics and analyzed for (A) CD16, (B) CD1b/c, (C) BDCA-3, (D) CD123, and (E) CD34 staining. Sort-purified Lin⁻HLA-DR⁺ cells were used to examine the relative intensity of HLA-DR and CD11c on each of the defined subsets: CD16 (F,K), CD1b/c (G,L), BDCA-3 (H,M), CD123 (I,N), and CD34 (J,O). Differences in fluorescence intensity for BDCA-3 (H,M) or CD11c (K-O) reflect the use of different fluorescent conjugates.

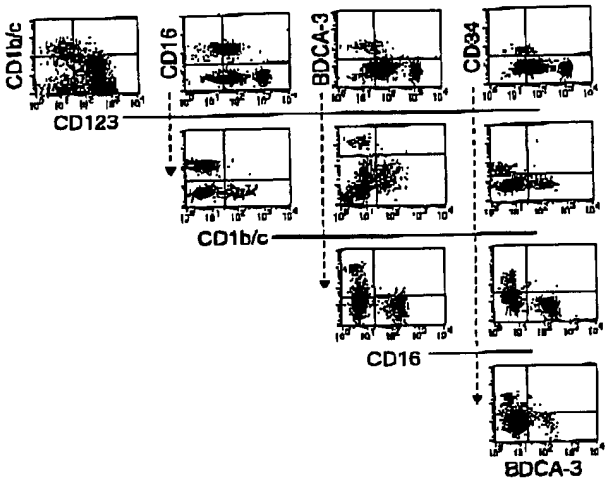


**Figure 3. The 5 phenotypically defined subsets in CD56-depleted Lin⁻ PBMC preparations represent nonoverlapping populations.** Sort-purified HLA-DR⁺ Lin⁻ cells were stained with various combinations of the defining mAb as indicated in the dot-plot representations.

BDCA-3, CD16, CD34, or CD123 showed that these subpopulations accounted for 94.1% of the HLA-DR⁺ cells. Furthermore, 93.8% of the CD11c⁺ population, which comprised 75.6% of the HLA-DR⁺ cells, was represented by 3 subsets: CD16⁺ (65.5%), CD1b/c⁺ (24.6%), and BDCA-3 (3.6%; Table 2). In these preparations, the CD123$^{hi}$ and CD34⁺ populations comprised the remaining CD11c⁻ cells, that is, 18.3% and 5.0% of HLA-DR⁺ cells, respectively. Thus, based on the discreet expression of CD123, CD1b/c, CD16, BDCA-3, and CD34, these Lin⁻HLA-DR⁺ preparations could be fractionated, almost in their entirety, into 5 distinct cell populations. The data in Table 2 suggest that, on average, 5.9% of Lin⁻HLA-DR⁺ cells do not belong to one of the subsets described. The mAbs to positively define these cells have not yet been identified and they were not characterized further in this study.

Having positively defined 5 populations, additional triple-labeling studies were undertaken to clarify that they were nonoverlapping subsets. Thus, sort-purified Lin⁻HLA-DR⁺ cells were stained with various combinations of the defining antibodies, and the restricted expression of each of these markers by the 5 Lin⁻HLA-DR⁺ populations was confirmed (Figure 3).

### The 5 Lin⁻HLA-DR⁺ PBMC subsets express unique phenotypes

We next examined the 5 Lin⁻HLA-DR⁺ populations for their expression of markers reported to be expressed by DC subsets. Lin⁻ preparations were labeled with FITC- or PE-SAM followed by HLA-DR–PE-Cy5, and the Lin⁻HLA-DR⁺ cells (ie, HLA-DR⁺ cells within R1 and R2, Figure 1) were sorted. They were then labeled with one of the 5 PE- or FITC-conjugated subset markers and one of a panel of PE- or FITC-conjugated mAbs or isotype-matched controls (Table 3). An analysis of CD14$^{hi}$ monocytes and immature MoDCs was also performed for comparative purposes. Of note, cutaneous lymphocyte antigen (CLA), a homing receptor for skin that has previously been reported to be widely expressed by DCs,[15,23] was not detected on the CD16⁺ population and only minimally expressed on the MoDCs. In contrast, all other populations expressed CLA either homogeneously (CD1b/c, BDCA-3, CD123) or heterogeneously (CD34 and CD14). Additionally, CD2 expression was limited to the CD1b/c⁺ population, a small and variable proportion of the CD123$^{hi}$ and CD34⁺ subsets, and a small subset of CD14$^{hi}$ monocytes. CD64 expression was restricted to the CD1b/c subset and the CD14$^{hi}$ monocytes. A small proportion of each Lin⁻HLA-DR⁺CD11c⁺ subpopulation expressed CD56, in contrast to CD7 expression, which was confined to a subset of the CD123$^{hi}$ population.

**Table 2. DC subset frequency**

| Donor no. | % of HLA-DR⁺ Lin⁺ cells | | | | | | Sum of 5 subsets |
|---|---|---|---|---|---|---|---|
| | CD16 | CD1b/c | BDCA-3 | CD123 | CD34 | CD11c | |
| 1 | 51.4 | 13.3 | 1 | 12.4 | 6.7 | 78.7 | 84.8 |
| 2 | 61.4 | 19.9 | 1.9 | 20 | 6.9 | 72.1 | 100.1 |
| 3 | 52.7 | 8.6 | 5.9 | 38.1 | 5.8 | 72.1 | 111.1 |
| 4 | 43.6 | 23.7 | 2.5 | 16.2 | 8.9 | 75.7 | 94.9 |
| 5 | 66.9 | 18.7 | 3.3 | 10.9 | 1.4 | 83.3 | 90.2 |
| 6 | 58.4 | 13.2 | 2 | 18.5 | 2.8 | 72.5 | 94.9 |
| 7 | 46.1 | 35 | 2 | 6.8 | 4.3 | 83.8 | 94.2 |
| 8 | 56 | 15.3 | 2.3 | 13.3 | 5.3 | ND | 92.2 |
| 9 | 30.6 | 19.9 | 3.2 | 28.3 | 2.8 | 68.4 | 84.8 |
| Mean | 49.6 | 18.6 | 2.7 | 18.3 | 5.0 | 75.6 | 94.1 |
| SD | 8.5 | 7.6 | 1.4 | 9.7 | 2.4 | 6.0 | 8.0 |
| Range | 31-66 | 9-24 | 1-6 | 7-38 | 1-9 | 66-94 | 85-111 |

### The 5 Lin⁻HLA-DR⁺ subsets have diverse CD85 (ILT) molecular profiles

Because DCs, either ex vivo or in vitro derived, express several family members of the CD85 family,[11] we examined the expression of some of the inhibitory members of this family (CD85j, k, d, a = ILT-2, -3, -4, and -5, respectively) by the Lin⁻HLA-DR⁺ subsets (Figure 4; Table 3).

CD85j (ILT-2/LIR1/MIR7) is expressed widely by leukocytes including myeloid DCs. CD85j expression by CD16-depleted Lin⁻ populations was initially analyzed by 3-color flow cytometry using HLA-DR, CD85-FITC (VMP55), and CD11c-PE or CD123-PE. Whereas all HLA-DR⁺CD123⁺⁺ cells and the majority of HLA-DR⁺CD11c⁺ cells expressed CD85j, a small population of CD11c⁺ cells lacked the expression of CD85j (Figure 4A). Further examination of the Lin⁻HLA-DR⁺ populations revealed high levels of CD85j on the CD16⁺ population and moderate levels of expression on the CD1b/c⁺ and CD123⁺ populations. Negligible expression of CD85j was detected on the BDCA-3⁺CD11c$^{lo}$ population. As suggested by our initial screening (Figure 4B), the Lin⁻HLA-DR⁺CD34⁺population was CD85j⁻.

**Table 3. Phenotype of HLA-DR$^+$ Lin$^-$ populations**

| | CD16 | CD1b/c | BDCA-3 | CD123$^{hi}$ | CD34 | CD14$^{hi}$ | MoDCs |
|---|---|---|---|---|---|---|---|
| HLA-DR | +/++* | ++ | ++ | +/++* | + | ++* | ++† |
| CD11c | +++ | +++ | ++ | - | - | +++ | +++ |
| CD33 | ++ | +++ | +++ | + | + | +++ | ++ |
| CD4 | + | + | + | ++ | - | + | ++ |
| CD2 | - | + | - | +† | +† | +† | + |
| CLA | - | ++ | ++ | + | +† | +† | +/- |
| CD7 | - | - | - | +† | - | +† | - |
| CD56 | +† | +† | +† | +† | NT | ++ | - |
| CD64 | - | + | - | - | - | + | - |
| CD62L | + | + | ++ | ++* | + | ++* | - |
| CD52 | +++ | ++ | ++ | ++ | ++ | ++ | - |
| ILT molecules | | | | | | | |
| ILT2/CD85j | ++ | + | - | + | - | + | ++ |
| ILT3/CD85k | + | ++ | +/- | ++ | - | + | + |
| ILT4/CD85d | ++ | +/- | - | - | - | + | +/- |
| ILT5/CD85a | + | + | - | - | - | + | + |
| C-type lectins | | | | | | | |
| DEC205/CD205 | + | ++ | +++ | ++ | ++ | ++ | + |
| MMR/CD206 | - | - | - | - | - | - | + |
| Langerin/CD207 surface | - | - | - | - | - | - | - |
| cytoplasmic | +/- | +/- | +/- | +/- | - | +/- | + (TGF-β) |
| DC-SIGN/CD209 | - | - | - | - | - | - | + |
| Costimulatory molecules | | | | | | | |
| CD40 | + | +* | ++ | +/- | + | + | ++ |
| CD80 | - | - | - | - | - | - | + |
| CD86 | ++ | + | + | + | - | + | + |

NT indicates not tested; TGF-β, positive staining after culture with TGF-β.
*Heterogeneous staining.
†Subset positive.

The expression of CD85k (ILT-3, mAb ZM3.8), CD85d (ILT-4, mAb 42D1), and CD85a (ILT-5, mAb 7H5) by the 5 populations is summarized in Table 3. Again, the CD34$^+$ population lacked expression of these 3 ILT molecules. The CD16 and CD1b/c subsets expressed varying levels of each. The BDCA-3 population expressed minimal levels of ILT-3, but none of the other molecules. The CD123$^{hi}$ population expressed ILT-3 at levels equivalent to the CD1b/c subset, but lacked expression of ILT-4 and ILT-5. Monocytes, as expected, expressed all the CD85 molecules tested at similar densities, whereas MoDCs expressed profiles intermediate to the CD16$^+$ and CD1b/c$^+$ Lin$^-$HLA-DR$^+$ populations.

### C-type lectin expression by the 5 Lin$^-$HLA-DR$^+$ subsets

DCs express several C-type lectin molecules, some of which may act as antigen uptake receptors. The expression of MMR (CD206), DEC-205 (CD205), DC-SIGN (CD209), and langerin (CD207) Lin$^-$HLA-DR$^+$ by the 5 Lin$^-$HLA-DR$^+$ subsets was examined in light of their potential to identify DC subpopulations. In contrast to MMR, DC-SIGN, and langerin, which were not detected on any of the Lin$^-$HLA-DR$^+$ subsets, DEC-205 was present on all the subsets (Table 3). Differential levels of DEC-205 expression were noted, with the BDCA-3$^+$ subset expressing the highest levels, followed by the CD123$^{hi}$, CD1b/c$^+$, and CD34$^+$ subsets, which expressed equivalent levels. The CD16$^+$ subset expressed the lowest levels. Fresh monocytes expressed DEC-205 at a level similar to the CD1b/c$^+$, CD123$^{hi}$, and CD34$^+$ subsets. Notably, MoDCs expressed lower levels of DEC-205 than the monocytes from which they were derived. Whereas MMR and DC-SIGN were absent from all Lin$^-$ populations (confirmed by reverse-transcription polymerase chain reaction, data not shown) and CD14$^{hi}$ monocytes, both of these lectins were expressed by MoDCs. Langerin was not found on the surface of any of the freshly purified DC populations examined and cytoplasmic levels were, at most, marginally positive (Table 3). Langerin was induced on a subset of CD11c$^+$Lin$^-$ cells after 18 hours of in vitro culture (in the absence of cytokines),[19] confirming the specificity of antibody staining, but the ability to discriminate the DC subsets was lost after in vitro culture and this aspect was not pursued further.

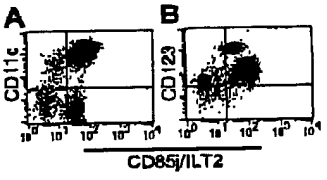


Figure 4. CD85j (ILT2) expression on CD56-depleted Lin$^-$HLA-DR$^+$ PBMCs. (A) CD11c labeling demonstrates heterogeneous CD85j expression within both the CD11c$^+$ and CD11c$^-$ compartments. (B) The CD123$^{hi}$ subset is CD85j$^+$, indicating that the CD34$^+$ subset lacks CD85j expression.

### Disparate CD40 and CD86 expression on freshly purified Lin$^-$HLA-DR$^+$ cells

To obtain insight into the activation status and costimulatory capacity of the 5 subsets, we examined their expression of CD40, CD80, and CD86 (Figure 5). The BDCA-3$^+$ population expressed significantly higher levels of CD40 than the other 2 CD11c$^+$ populations. A broad range of CD40 expression by the CD1b/c$^+$ population was noted compared to the expression by the CD16$^+$ and BDCA-3$^+$ populations, possibly reflecting a continuum of activation. CD86 expression did not correlate with CD40. Thus, the highest level of CD86 expression was detected on the CD16$^+$ subset, whereas the CD1b/c$^+$ and BDCA-3$^+$ populations expressed

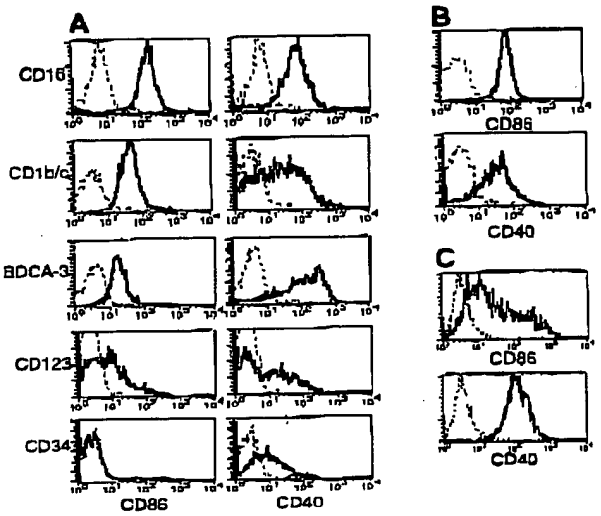


**Figure 5. Costimulatory molecule expression by Lin⁻HLA-DR⁺ subsets, monocytes, and MoDCs.** CD56-depleted Lin⁻ PBMC preparations were stained with HLA-DR, one of the subset-defining mAbs, and one of PE-conjugated CD86, CD40, or negative control mAbs. Histograms representing CD86 and CD40 expression (solid lines) by (A) CD56-depleted HLA-DR⁺Lin⁻ subsets is compared to that of (B) monocytes and (C) MoDCs. Negative control antibody staining is indicated by a dashed line.

lower levels. By comparison, monocytes expressed CD86 and CD40 (Figure 5B) at levels equivalent to the CD1b/c⁺ and CD16⁺ subsets, respectively. None of the Lin⁻HLA-DR⁺ subsets or monocytes expressed detectable levels of cell surface CD80, in contrast to MoDCs, which uniformly expressed low levels (Table 3).

### Decreased viability of the CD123⁺ and CD16⁺ subsets following in vitro culture

Differences between the CD11c⁺ and CD123⁺ subsets in their survival in vitro have been described.[24] Therefore, we examined the survival of the DC subsets following 18 hours of culture. Cultured CD11c⁺ DCs have been observed to up-regulate cell surface CD123, such that gating CD11c⁺ and CD11c⁻ populations becomes difficult. Furthermore, following 18 hours of culture, BDCA-3 was expressed by an increased proportion of CD11c⁺ cells and was induced on a subset of CD123⁺ cells.[18] Thus, to facilitate subset tracking in these studies, Lin⁻CD11c⁺ and CD11c⁻ subsets were sorted prior to culturing. Following 18 hours in the presence of GM-CSF and IL-3, or allogeneic T cells, the survival of the CD11c⁺CD16⁻ (CD1b/c⁺ and BDCA-3⁺ populations), CD11c⁺CD16⁺, CD11c⁻CD34⁻ (CD123⁺ population) and CD11c⁻CD34⁺ populations was examined. Even in the presence of GM-CSF and IL-3, cytokines reported to increase the survival of the CD11c⁺ and CD123⁺ cells, respectively,[24] significant cell death occurred in both the CD11c⁺ and CD11c⁻ cultures. Comparison of the relative proportion of CD16⁺ and CD34⁺ cells in these cultures indicated that the CD11c⁺CD16⁺ and CD123⁺ populations had the poorest survival (Figure 6A). Thus, whereas the freshly sorted CD11c⁺ preparations contained 68% (± 6.6% SD; n = 4) CD16⁺ cells, following culture, this decreased to 31% (± 6.7%; n = 4) of viable (based on forward- and side-scatter characteristics) CD11c⁺ cells. To demonstrate that the loss of CD16⁺ cells was due to cell death rather than the loss of cell surface CD16 expression, CD11c⁺ (CD16⁺ inclusive) and CD11c⁺CD16⁻ populations were sort purified and cultured as described above. As can be seen in Figure 6B, the CD11c⁺ (CD16⁺ inclusive) cultures contained an increased proportion of PI⁺ dead cells (46%) as compared to the CD11c⁺CD16⁻ cultures (13%). Sort-purified CD11c⁺CD16⁺ cells were also examined and exhibited equally poor survival in culture (data not shown). Similarly, the relative frequency of CD34⁺ cells in CD11c⁻ populations following 18 hours culture increased (from 28% to 71%) reflecting a proportionately greater decrease in the number of CD123⁺ cells. Addition of allogeneic T cells to the DC cultures had no effect on the relative proportion of any of the subsets examined (data not shown). Thus, as previously documented, DCs survive poorly in vitro, with the CD123⁺ and the CD11c⁺CD16⁺ subsets having the poorest survival rate of the 4 subsets examined.

### In vitro induction of activation-associated molecules on Lin⁻HLA-DR⁺ subsets

Having established that the 5 subsets exhibited distinct molecular profiles, and that, at least a proportion of each of these subsets survived in vitro, we compared their relative expression of the activation-associated antigens CMRF-44, CMRF-56, and CD83 following in vitro culture. Lin⁻CD11c⁺ (including CD16⁺) and CD11c⁻ subsets were sorted and cultured as described above, and the expression of CMRF-44, CMRF-56, and CD83 was examined on the CD11c⁺CD16⁻ and CD11c⁺CD16⁺ subsets (Figure 7A) and the CD11c⁻CD34⁺ (ie. CD34⁺) and CD11c⁻CD34⁻ (ie. CD123⁺) populations (Figure 7B). The cultured CD11c⁺CD16⁻ cells expressed uniformly high levels of each of CMRF-44, CMRF-56, and CD83. The level of expression of these antigens was not increased further on this population following coculture with allogeneic T cells. CMRF-44, CMRF-56, and CD83 were induced on the CD11c⁺CD16⁺ population; however, the levels of expression of CMRF-44 and CMRF-56 were distinctly lower than that of the CD11c⁺CD16⁻ subset, and only a proportion of the CD16⁺ cells expressed CD83. Minimal additional increases in CMRF-44 and CMRF-56 staining of both the CD16⁻ and CD16⁺

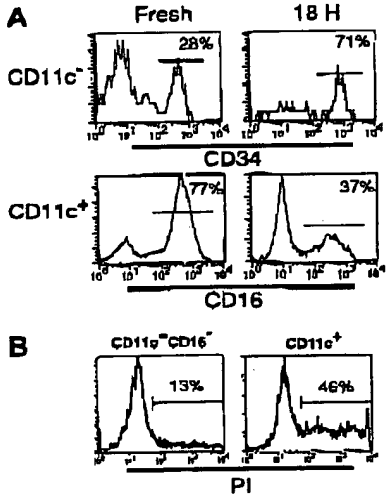


**Figure 6. Differential survival of Lin⁻HLA-DR⁺ PBMC subpopulations.** (A) The frequency of CD34⁺ or CD16⁺ cells was examined in freshly sorted or cultured (18 hours with GM-CSF and IL-3) Lin⁻ CD11c⁻ or CD11c⁺ cells, respectively. The preferential survival of CD34⁺ cells over CD123ᴵᴺ (top panel) and reduced frequency of the CD11c⁺CD16⁺ subset (bottom panel) following culture were noted. (B) The frequency of dead cells was examined in 18-hour cultures of sort-purified CD11c⁺CD16⁻ and CD11c⁺ (CD16⁺ inclusive) populations. An increase in the percentage of PI⁺ (dead) cells was associated with the CD11c⁺ (CD16⁺ inclusive) cultures.

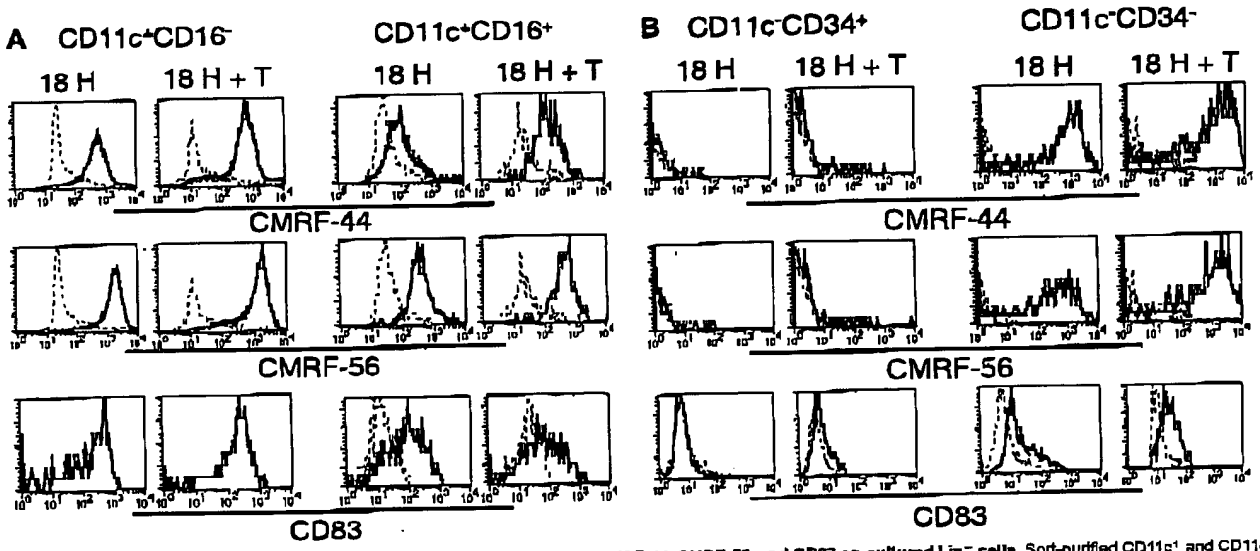


**Figure 7. Induction of the DC-associated differentiation/activation antigens CMRF-44, CMRF-56, and CD83 on cultured Lin⁻ cells.** Sort-purified CD11c$^+$ and CD11c$^-$ populations were cultured for 18 hours in the presence of GM-CSF and IL-3 with or without allogeneic T lymphocytes. Following culture, cells were harvested and stained with biotinylated CMRF-44 or CMRF-56, or purified CD83 mAb followed by biotinylated anti–mouse IgG. Biotinylated antibody was detected with PE- or PE-Cy5–conjugated streptavidin, in conjunction with PE-Cy5– or PE-conjugated CD16 or CD34 for the (A) CD11c$^+$ and (B) CD11c$^-$ populations, respectively. CD11c$^+$CD16$^-$, CD11c$^+$CD16$^+$, CD11c$^-$CD34$^+$, and CD11c$^-$CD34$^-$ populations were gated and examined for their expression of activation antigens (solid lines). Dashed lines represent negative control antibody staining.

subsets resulted after coculture with T cells. Cultured CD123$^+$ (CD11c$^-$CD34$^-$) cells expressed CMRF-44 and CMRF-56 at levels comparable to the CD11c$^+$CD16$^-$ population, whereas CD83 expression was much less. However, coculture with allogeneic T cells induced higher levels of all 3 molecules on the CD123$^+$ population. Finally, only a very small proportion of CD34$^+$ cells was induced to express CMRF-44 or CMRF-56, which was slightly increased by T-cell coculture. CD83 was not detected on the CD34$^+$ cells in either culture condition.

### Allostimulatory capacity of Lin⁻HLA-DR$^+$ subsets

A defining property of DCs is their potent antigen-presenting function. We, therefore, examined the capacity of the 5 Lin$^-$ subsets to induce T-cell proliferation in allogeneic MLRs. Monocytes and MoDCs were included as reference populations. For comparative purposes, the CD1b/c$^+$ population was used as the internal standard for each MLR. Each of the cell populations tested exhibited some degree of allostimulatory capacity although this was minimal for monocytes (Figure 8). Comparison of the blood Lin$^-$HLA-DR$^+$ populations demonstrated clear differences in their allostimulatory capacity; they were ranked CD1b/c > CD16 > BDCA-3 > CD123 > CD34. The stimulator dose/T-cell proliferative response curve for the BDCA-3 subset differed significantly from the others, with an apparent early plateau. The CD123$^+$ populations were less effective stimulators of the MLR and the CD34$^+$ population was, as previously reported, a weak but definite stimulator of an allogeneic MLR.

## Discussion

It is clear from recent differences in data reported in the literature[10,12,15] and from data presented at the 7th LDAW[16,19] that the cellular constitution of human blood DC preparations varies considerably. The main factors likely to contribute are the mAb mixtures used to select Lin$^-$ cells, the immunoselection methodology, and the gating criteria used in subsequent flow cytometric analysis. We undertook this analysis of the composition of human Lin$^-$ or putative DC preparations in an effort to define the cellular heterogeneity and thus provide a basis to compare preparations, methodology, and results from different laboratories. We prepared Lin$^-$ cells by depleting with CD3, CD14, CD19, CD11b, and CD16 or CD56 mAbs but used CD56 in preference, because this permitted the examination of a Lin$^-$HLA-DR$^+$ CD16$^+$ population,

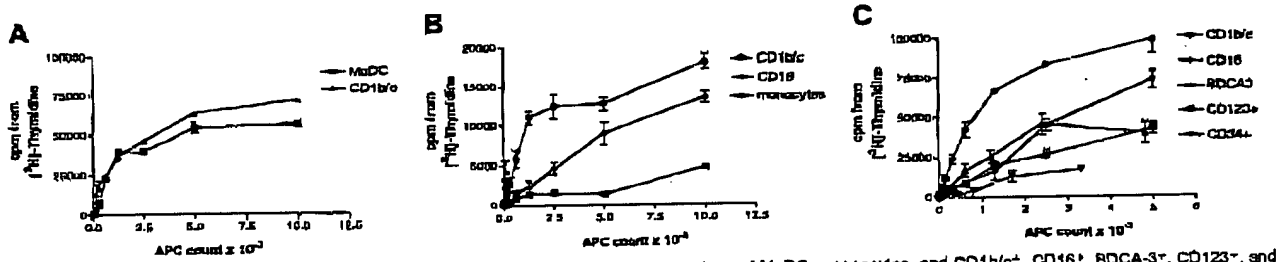


**Figure 8. Allostimulatory capacity of sort purified Lin⁻ cell subpopulations.** Varying numbers of MoDCs, monocytes, and CD1b/c$^+$, CD16$^+$, BDCA-3$^+$, CD123$^+$, and CD34$^+$ Lin$^-$ cells were cultured for 5 days in a 96-well plate with $10^5$ allogeneic normal peripheral blood T cells per round-bottom well. T-cell proliferation was assessed by addition of tritiated thymidine. Results are expressed as means ± SEM of triplicate wells. Three separate representative experiments (A-C) are shown. Data are representative of a minimum of 3 experiments for each subset compared to the CD1b/c$^+$ population.

which has been reported to exhibit DC characteristics. We used a panel of markers previously reported to be expressed by DCs or cells within Lin$^-$ preparations to analyze the Lin$^-$HLA-DR$^+$ population. This analysis identified 5 distinct nonoverlapping subsets that we identified as CD123$^{hi}$, CD1b/c$^+$, CD16$^+$, BDCA-3$^+$, and CD34$^+$. A more extensive phenotypic analysis of these subsets including costimulatory molecules, ILT molecules, C-type lectins, and others demonstrated a unique molecular phenotype for each of the 5 subsets. These differed from the molecular phenotypes obtained for circulating CD14$^{hi}$ monocytes or MoDCs. Furthermore, the culture requirements, activation potential, and allostimulatory capacity differed among these 5 Lin$^-$ populations. Having phenotypically defined these 5 discrete subsets in blood, the substantial task of assigning specialized functional roles or stages of development to these subsets remains to be addressed. We have demonstrated the basic allostimulatory capacity of each subset, but a myriad of additional functional assays are required to compare antigen uptake, cross-presentation, cytokine secretion, presentation to and activation of B and T lymphocytes (CD4 versus CD8, memory versus naive, $T_H1$ versus $T_H2$ polarization), and so forth.

The CD11c$^+$ or myeloid blood DC population has been noted to be heterogeneous in our own[16,25] and other studies.[8,18] We show here that it includes the CD16$^+$, CD1b/c$^+$, and BDCA-3$^+$ subpopulations. The CD16$^+$ population comprised a large but variable (40%-80%) proportion of the CD11c$^+$ subset. It expressed low levels of CD14 and CD33, was the only subset to lack CLA expression, and had heterogeneous but generally lower levels of cell surface HLA-DR. Curiously, the CD16$^+$ population had the highest levels of CD86 and relatively high levels of CD40. An important feature of this cell population was its apparently poor viability in tissue culture with only 50% surviving 12 to 18 hours in 10% FCS/RPMI-1640, even in the presence of GM-CSF and IL-3. The CD1b/c$^+$ population represents 20% to 50% of the CD11c$^+$ population and was noted to express the highest levels of CD11c. It expresses low to negligible levels of CD14 but is clearly CD33$^+$ and universally expresses high levels of cell surface HLA-DR. The correlation between increasing CD1b/c expression and increasing CD11c and HLA-DR expression in conjunction with heterogeneous CD40 expression is indicative of a population of cells undergoing differentiation. The BDCA-3 subpopulation is much smaller and accounts for 2% to 3% of the CD11c$^+$ cells. Interestingly, its CD11c expression was consistently at the lower end of the range of the CD11c$^+$ population. It lacked CD14 but was again clearly CD33$^+$ with moderate to high levels of cell surface HLA-DR. Notably, these BDCA-3$^+$ cells had lower levels of CD86 but expressed the highest levels of CD40 and DEC-205.

As previously reported,[9,10,25] the lymphoid or CD123$^{hi}$ DC population was readily distinguished from the myeloid CD11c$^+$ DC population. However, we note that the CD11c$^+$ population is unequivocally weakly CD123$^+$ and that the expression of CD123 increases on in vitro culture, so that gating using this marker needs to be done carefully. Some heterogeneity in the CD123$^{hi}$ population was noted with differential expression of HLA-DR, CD40, CD2, and CD7.

These studies emphasize the fact that Lin$^-$HLA-DR$^+$ preparations may include other cell populations, which may or may not be classified as DCs or DC precursors. The CD34$^+$ population presumably reflects, in part, the low-level circulating population of hematopoietic progenitors.[26] Some or all of these have the capacity to differentiate into DCs.[27,28] and it has been known for some time that these cells have allostimulatory potential.[29] Our data in Table 2 suggest that one or more additional subsets of Lin$^-$HLA-DR$^+$ cells exist in blood. These will be CD123$^{-/lo}$ and CD34$^-$ and either CD11c$^+$ or CD11c$^-$. We did not characterize them further in this study because of the small numbers and lack of defining antibodies. We also note, in passing, that all 5 Lin$^-$HLA-DR$^+$ populations including the CD34$^+$ population express CD52, a fact that may be relevant to interpreting the immunosuppressive effect of CD52 therapy in allogeneic transplantation.[29] The low level of staining on CD34$^+$ hematopoietic stem cells, or the self-renewing subset, may account for the apparent lack of influence on engraftment.

In terms of the function of these subsets, we investigated their expression of several relevant molecules and the classic definition of DC function—their ability to stimulate in an allogeneic MLR. Both the CD123$^{hi}$ DCs and the BDCA-3$^+$ subpopulation of CD11c$^+$ DCs express high levels of CD62L. It has been suggested that CD62L may mediate the migration of the CD123$^+$ population directly from the blood via high endothelial venules (HEVs) into lymphoid tissues,[30,31] thus explaining the relative paucity of this population in the normal peripheral tissues. It is possible that the BDCA-3 population behaves similarly, but the tissue distribution of this subset is only now undergoing analysis. A number of C-type lectins were investigated in relation to their potential as antigen uptake receptors. It appears that all 5 Lin$^-$HLA-DR$^+$ populations either lack or require a signaling event to induce surface expression of CD206, CD207, or CD209. CD206 is not induced on blood DCs but is on MoDCs.[32] The same is true of CD209,[33] which has recently been described with DC-SIGNR and CD23 on tissue macrophages.[34] The induction of langerin (CD207) on in vitro–derived DCs requires the presence of transforming growth factor β (TGF-β).[35] CD11c$^+$/CD1a$^+$ blood DCs have been claimed to be direct precursors of the langerin-expressing LCs.[15] The CD1 mAb used in that study was the BI35 clone but subsequent work during the 7th LDAW showed it to recognize CD1b/c rather than CD1a.[16] Insignificant levels of cytoplasmic langerin were detected in the putative LC precursor, CD1b/c$^+$ subset, and in the other subsets (Table 3). The broad expression of CD205 (DEC-205) as a potential antigen-loading receptor on all the cells is interesting. Our own unpublished and other data[36] suggests DEC-205 does load antigen into antigen-presenting cells, but it may have other functions on other cells, as suggested by its presence on CD34$^+$ cells. There is also increasing awareness that DC molecular phenotype and function are highly regulated and these may differ according to the stimuli.[37] The different CD85 molecule profiles on the 5 subsets is therefore highly relevant, given their likely contribution to both up- and down-regulation of intracellular signaling pathways in both DC and other leukocyte populations.[11,30]

The MLR studies produced unequivocal results. The CD1b/c population invariably expressed high levels of the CD83, CMRF-44, and CMRF-56 antigens, which have been associated with DC differentiation or activation. Consistent with this, the CD1b/c subset was invariably the most potent allostimulatory cell population with the CD16$^+$ population the next most effective stimulators. Curiously enough, these results were independent of the level of expression of the classic costimulator molecules CD80/CD86 and this may suggest that other costimulatory molecules such as OX40L, RANKL, or 41BBL may be involved. We confirmed that CD123$^{hi}$ DCs stimulate an allogeneic MLR[9] as does the CD34$^+$ cell population[29] although the latter cells were the least effective stimulators in the absence of any prior in vitro differentiation. Clearly, further analysis of a number of different functional properties (including susceptibility to CD85-mediated inhibition)

of these 5 potential DC subpopulations is required. The practicalities of this are daunting, but the major molecular and functional differences outlined in this work argue that each population may have to be addressed individually. It is even possible that certain subpopulations will be preferred for certain therapeutic applications. In our hands, the blood CD11c$^+$ DCs[38] and the CMRF-56$^+$ DCs (Lopez et al, submitted manuscript) stimulate strong in vitro blood primary T-lymphocyte responses.

Finally, although it is tempting to try and assign these CD11c$^+$ subsets to a theoretical DC differentiation/activation pathway, we think it is premature to do so. The CD11c$^+$CD1b/c$^+$ subset appears the most activated phenotypically and functionally but whether it is derived from a CD14$^+$ precursor as previously suggested[15,16] requires confirmation. Although the relationships of these subpopulations to accepted hematologic pathways needs to be established, no direct correlation with MoDCs was observed. The results nonetheless bring some clarity to the field and the definition of these Lin$^-$HLA-DR$^+$ subsets provides an opportunity to standardize the populations for further molecular and possibly even therapeutic functional studies.

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# Human Dendritic Cell Differentiation Pathway From CD34+ Hematopoietic Precursor Cells

By Michelle Rosenzwajg, Bruno Canque, and Jean Claude Gluckman

The most effective antigen-processing cells for T lymphocytes are dendritic cells (DCs), the differentiation pathway of which, however, is incompletely characterized. We examined here how DCs differentiated from human cord blood CD34+ progenitor cells cultured with granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, and stem cell factor. After 5 days, 2 of 3 nonadherent cells were CD13hiHLA-DRhiCD14-, half of them were also CD14+, and ≤10% were CD1a+. When day-5 sorted CD13hiCD1a- and CD13lo cells were further cultured, CD1a+ cells appeared in the already CD13hi population, whereas CD13hi cells, a minority of which rapidly became CD1a+, emerged from the CD13lo population. By day 12, still 66% of built cells in suspension were CD13hi, most of which displayed high forward and side scatters of large granular cells. Half of CD13hi cells were CD1a+. All CD13hi cells expressed to the same extent DR, CD4, costimulatory and adhesion molecules, and various amounts of CD14. CD1a+ cells stimulated allogeneic lymphocytes more than CD13hiCD1a- cells and, although they were CD14-, both cell types were nonspecific esterase-negative nonphagocytic cells and were stronger mixed leukocyte reaction stimulators than were their macrophage counterparts. Eventually, the percentage of CD1a+ cells decreased. However, typical CD1a+ DCs still emerged in culture of sorted day-12 CD13hiCD1a- cells, and adding interleukin-4 to built cultures at that time led to the persistence of the CD1a+ population while diminishing CD14 expression. Thus, this system results first in the differentiation of CD13lo precursors that strongly express DR and CD4, from which more mature CD1a+ DCs continuously differentiate all along the culture period.

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DENDRITIC CELLS (DCs) are the most potent antigen-presenting cells (APCs).[1-4] Only they can prime naive T-helper lymphocytes, whereas macrophages or activated B cells stimulate only primed cells[2,5-7] and also can elicit cytotoxic T lymphocyte responses to soluble antigens.[8-10] DCs are present in most tissues, in which they display different maturation states according to the local microenvironment[3]; eg, they are known as Langerhans cells (LCs) in the skin, as interdigitating cells in lymphoid tissues, and as veiled cells in the lymph and blood.[2,11]

Characterization of DCs in humans has been confounded both by the lack of exclusive lineage-specific markers and by their low numbers and consequent difficult isolation as pure populations; eg, they represent ≤1% of mononuclear cells (MNCs) in the readily accessible peripheral blood.[2,3,7,12,13] Nonetheless, some common phenotypic pattern appears from recent studies of different, mainly blood-derived and skin DC populations[7,12,14-17]: (1) the dendritic morphology, (2) the low phagocytic activity, (3) the high membrane density of HLA class II molecules and of accessory molecules, (4) the expression of CD1a, and (5) the strong capacity to stimulate allogeneic T lymphocytes.[3]

The differentiation pathway of this apparently heterogeneous cell population is still incompletely known in humans, and its lineage relationship with other bone marrow-derived cells, especially monocyte/macrophages, is questioned if only because the in vitro generation of mixed colonies of DCs, macrophages, and granulocytes from single CD34+ hematopoietic precursors appears common.[18,19]

The possibility to generate human DCs in vitro from bone marrow, cord blood, or even adult peripheral blood precursors[18-22] under the influence of combinations of different growth factors (granulocyte-macrophage colony-stimulating factor [GM-CSF], tumor necrosis factor-α [TNF-α], stem cell factor [SCF], and interleukin-4 [IL-4]) should allow us to solve many questions regarding DC differentiation and functions as well as their relationship with cells of other lineages.[23] We used here a recently described technique[19,20] to generate DCs in vitro from purified cord blood CD34+ stem/progenitor cells to analyze the DC in vitro differentiation pathway.

## MATERIALS AND METHODS

*Cord blood CD34+ cell isolation and culture.* Normal cord blood was obtained from Laboratoire Senders, hôpital Saint-Vincent de Paul (Paris, France), according to institutional guidelines. The cell suspension, diluted 1:1 in RPMI 1640 (GIBCO BRL, Paisley, UK), was centrifuged on Ficoll-Paque (Pharmacia, Uppsala, Sweden). After washing in phosphate-buffered saline (PBS), 2% fetal calf serum (FCS; GIBCO BRL), MNCs ($1 \times 10^8$/mL in PBS, 2% FCS) were incubated for 30 minutes at 4°C with 1:20 diluted fluorescein isothiocyanate (FITC)-labeled CD34 monoclonal antibody (MoAb) HPCA-2 (Becton Dickinson, Mountain View, CA), and CD34+ cells were sorted by flow cytometry (FACStar Plus; Becton Dickinson) at 2,000 to 3,000 cells/s from a $5 \times 10^6$/mL suspension. Gates were drawn on the two-color cytogram within a dual-parameter cytogram of side versus forward light scatter. Equations were set to positively sort cells satisfying both gates. This procedure yielded 94% ± 3% pure viable CD34+ cells.

Alternatively, CD34+ cells were positively purified with CD34 MoAb 561-coated M-450 Dynabeads (Dynal, Oslo, Norway), incubating $4 \times 10^7$ beads with 2.5 to $5 \times 10^7$ cells/mL for 45 minutes at 4°C. Rosetted cells were seperated with a magnet, and beads were detached from the cells with DETACHaBEAD CD34 (Dynal) according to the manufacturer's instructions. This yielded 87% ± 13% pure viable CD34+ cells.

CD34+-enriched cells (2 to $5 \times 10^4$/mL) were cultured in 6-well

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*Submitted May 5, 1996; accepted August 25, 1995.*

*Supported by the Centre National de la Recherche Scientifique (ERS 107), Université Paris 6, the Agence Nationale de Recherche sur le SIDA, and the Association de Recherche sur les Déficits Immunitaires Viro-Induits (Paris, France).*

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*0006-4971/96/8702-0019$3.00/0*

Table 1. List of the MoAbs Used in the Study

| Marker | MoAb | Source |
|---|---|---|
| CD34 | HPCA-2 | Becton Dickinson |
| CD1a | T6/OKT6 | Coulter/Ortho |
| | Leu-2a/Leu-3a | |
| CD4 | + b | Becton Dickinson |
| | IOT4a | Immunotech |
| Myeloid markers | | |
| | | Becton Dickinson/ |
| CD13 | Leu-M7/IOM13 | Immunotech |
| CD14 | Leu-M3/My4 | Becton Dickinson/Coulter |
| CD15 | Leu-M1 | Becton Dickinson |
| CD33 | Leu-M9 | Becton Dickinson |
| Integrins/adhesins | | |
| CD18 | IOT18 | Immunotech |
| CD29 | IOT29 | Immunotech |
| CD44 | IOL44 | Immunotech |
| CD54 | Leu-54 | Becton Dickinson |
| CD58 | IOL58 | Immunotech |
| Costimulatory molecules | | |
| CD40 | | Pharmingen |
| CD80 | | Becton Dickinson |
| CD86 | | Pharmingen |
| MHC class II | | |
| HLA-DR | | Becton Dickinson |
| HLA-DQ | Leu-10 | Becton Dickinson |
| T-cell, B-cell, and NK cell markers | | |
| CD3 | T3 | Coulter |
| CD19 | Leu-12 | Becton Dickinson |
| CD56 | Leu-19 | Becton Dickinson |
| Miscellaneous | | |
| CD38 | Leu-17 | Becton Dickinson |

plates (ATGC, Noisy le Grand, France) at 37°C in humidified 5% $CO_2$ in RPMI 1640, 10% FCS, 1% glutamine, and 1% antibiotics (GIBCO BRL), supplemented with recombinant human growth factors (Genzyme, Cambridge, MA) as described[10,23,24]: human granulocyte-macrophage colony-stimulating factor (huGM-CSF; 200 U/mL), human tumor necrosis factor-α (huTNF-α; 50 U/mL), with or without human stem cell factor (huSCF; 50 ng/mL) and/or human interleukin-4 (huIL-4; 50 ng/mL). Cultures were split on day 5 and then every 3 to 4 days.

*Flow cytometry cell surface marker analysis and secondary sorting.* Cells were incubated for 30 minutes at 4°C with FITC- and/or phycoerythrin (PE)-conjugated mouse MoAbs (1:100 final) in PBS, 2% FCS. After washing, cells were resuspend in PBS and 1% paraformaldehyde and analyzed with a FACScan (Becton Dickinson). The MoAbs used are listed in Table 1. Negative controls were irrelevant MoAbs (Immunotech, Marseille, France).

For sorting, day-5 or day-12 cultured cells (5 to 10 × $10^6$/mL in PBS, 2% FCS) were washed, incubated at 4°C for 30 minutes with FITC-conjugated anti-CD1a and PE-conjugated anti-CD13 MoAbs, and washed again. Cells were then sorted with the FACStar Plus according to both CD13 and CD1a expression.

*Immunocytochemical staining and other assays.* Air-dried cytospin preparations were fixed for 5 minutes in 25% chloroform/acetone and kept at −70°C or they were fixed in PBS, 0.2% glutaraldehyde, and 1% formaldehyde and kept for few days at 4°C in PBS, 2% FCS. After thawing, slides were fixed again, air-dried, and rehydrated for 2 minutes with Tris-buffered saline (TBS), 0.5% bovine serum albumin (Sigma, St Louis, MO), 0.5% AB serum, 0.02% NP40, and 0.3% $H_2O_2$. After washing in TBS, slides were incubated for 2 hours at 20°C or overnight at 4°C with antibody. After two washes, slides were incubated for 45 minutes with 1:100 diluted alcaline phosphatase-labeled antirabbit or peroxydase-labeled antimouse antibodies (Dako, Glostrup, Denmark) and then with Fast Red TR/Naphtol AS-MX or diaminobenzidine (Sigma). Reactions were stopped and slides were counterstained with Harris' hematoxilin (Sigma) and mounted. The MoAbs used were anti-CD1a (L544, gift from L. Boumsell), anti-DR (Dako), BB1-B7 (Becton Dickinson), anti-CD40 (IOB40; Immunotech), and anti-S100 (Dako).

Nonspecific esterase staining was performed using naphthyl-butyrate (Sigma) as substrate. To assay phagocytosis, cells were incubated with 1.16-μ latex beads (Sigma) for 30 minutes at 37°C. After washing, cells were analyzed by light microscopy. Cells having ingested greater than 3 beads were scored as positive.

*Mixed leukocyte reaction (MLR).* Ficoll-Paque–separated adult peripheral blood MNCs (5 × $10^4$/100 μL) were cultured for 6 days in RPMI 1640, 10% FCS, 1% glutamine, and 1% antibiotics in 96-well U-bottomed culture microplates (Costar, Cambridge, MA) as responder cells to up to 1 × $10^4$/100 μL 30-Gy irradiated stimulator cells, which were different populations of the $CD34^+$-derived cells. [$^3$H]thymidine (Amersham, Amersham, UK) incorporation was measured after an 8-hour pulse with 1 μCi/well. Results are shown as mean counts per minute (cpm) of triplicates.

## RESULTS

*Growth of cultured cells differentiating from $CD34^+$ precursors.* In a first set of experiments, 5 × $10^4$ to 1.5 × $10^5$ $CD34^+$ cells purified from 0.9 to 4 × $10^8$ cord blood MNCs were cultured in the presence of huGM-CSF and huTNF-α.[18-20] This led to steadily increasing numbers of cells in suspension, which reached up to $10^7$ on day 12 and plateaued or decreased thereafter. To increase yields, cells were also cultured with huSCF added to huGM-CSF and huTNF-α from the start.[24,25] After 12 days, the cells had expanded 7- ± 3-fold in the absence of huSCF relative to 45- ± 27-fold when huSCF was present, without affecting the phenotype of the recovered cells or the proportion of DCs (data not shown). After 5 days, huSCF did not influence cell growth, and it could be discontinued. Unless otherwise stated, the data shown here were obtained with cells grown under the latter conditions.

As described,[19] either culture condition led to occurrence of small adherent aggregates with peripheral cells displaying typical DC morphology during the first 5 days of culture. Aggregate numbers increased and they enlarged until day 12, releasing DCs in the medium, while a few adherent macrophage-like cells also progressively appeared (data not shown).

*Early phenotypic evolution of the cells in suspension.* The bulk of initially purified $CD34^+$ cells were $CD38^-$ $DR^+CD13^{lo}CD33^{lo}CD1a^-$; according to the MoAb, these cells appeared as $CD4^-$ (IOT4a) or $CD4^{lo}$ (Leu-3a, Leu3a+b; data not shown).[26]

After 5 days, less than 10% $CD34^+$ cells remained in culture. Two nonadherent cell populations were distinguished according to CD13 expression; as averaged by quantitative analysis from seven different experiments, 32% ± 7% of cells were $CD13^{lo}$ and 65% ± 8% were $CD13^{hi}$ (Fig 1A). The $CD13^{lo}$ cells were $CD4^-CD14^-CD1a^-$, $DR^+$, or $DR^-$; $CD13^{hi}$ cells were $DR^{hi}CD4^{lo}$, with about 50% also

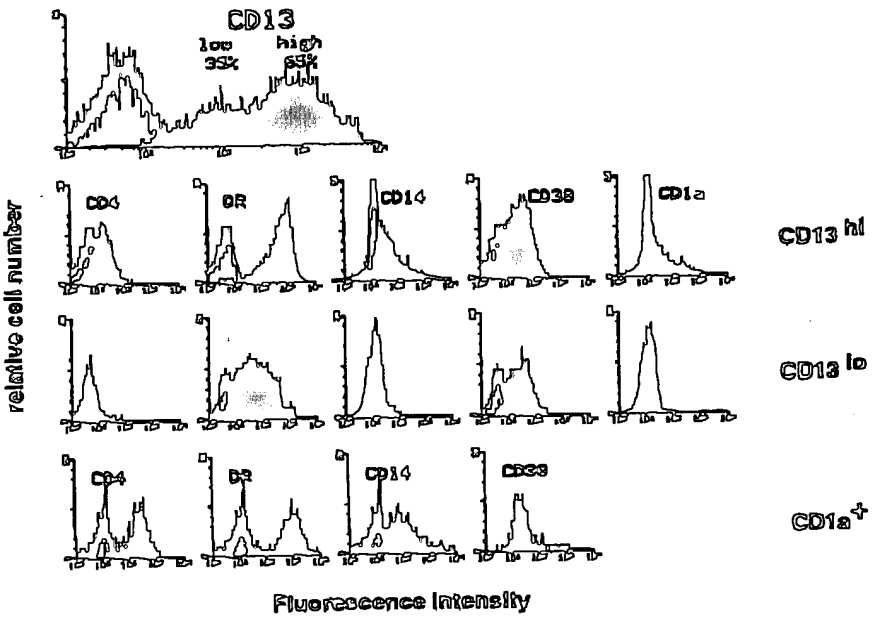


Fig 1. Phenotypic flow cytometry characterization of day-5 cultured cells. CD13 labeling allows us to distinguish the relative proportion of $CD13^{hi}$ and $CD13^{lo}$ cells (A). Cell surface marker analysis of $CD13^{hi}$ versus $CD13^{lo}$ cells (B). Cells were labeled both with a CD13-PE and an FITC-conjugated antibody against the indicated marker. Cells were gated according to the PE low and high fluorescence intensity. FITC fluorescence was then analyzed. Similar analysis was performed for $CD1a^{+}$ cells, but here gating was performed according to the positive CD1a-FITC labeling and the PE fluorescence was analyzed (C). Open histograms are negative controls that correspond to the green fluorescence of CD13-PE–labeled cells (B) or to the red fluorescence of CD1a-FITC–labeled cells (C). Solid histograms correspond to FITC-labeled (B) or PE-labeled (C) relevant MoAb.

being $CD14^{+}$ (Fig 1B). There were then 9% ± 4% $CD1a^{+}$ cells, which were $CD13^{hi}$, $CD4^{+}$, $DR^{+}$, $CD14^{+}$ (Fig 1C); they were $CD38^{-}$, whereas the other cells were either $CD38^{-}$ or $CD38^{+}$, with $CD13^{lo}$ cells expressing more CD38 (Fig 1B and C).

By day 12, two populations of cells in suspension were individualized according to size and granularity. One displayed low light scatter signals characteristic of small agranular cells (R1: 75% ± 7% of cells; n = 13). The other had high scatter and autofluorescence signals of large granular cells (R2; Fig 2A). Whereas all R2 cells were $CD13^{hi}$ and 75% ± 14% of them were $CD1a^{+}$, only 43% ± 14% of R1 cells were $CD13^{hi}$, among which 8% ± 4% were $CD1a^{+}$. Altogether, whether large or small, $CD13^{hi}$ cells still represented 66% ± 13% of the cells and half of them (36% ± 14%) were $CD1a^{+}$. All $CD13^{hi}$ cells expressed to the same extent CD4, DR, DQ, as well as adhesion molecules CD18, CD29, CD44, CD54 (ICAM-1), and CD58 (LFA-3) and costimulatory molecules CD40, CD80 (B7/BB1), and CD86 (B70/B7-2)[27]; CD14 was present on these cells but its expression was variable, with some differences in intensity according to the MoAb used (My4 or LeuM3); $CD13^{hi}$ cells did not express CD15 (Fig 2B).

All day-12 cells were $CD33^{+}$ but B-cell, T-cell, and NK cell markers (CD19, CD3, and CD56) were not detected (data not shown). $CD13^{-/lo}$ cells ($CD33^{+}DR^{-}CD4^{-}CD14^{-}CD15^{+/-}$), which belonged to the granulocyte lineage, were found only among R1 cells (data not shown) and were not investigated further here.

Taken together, these findings suggest that, but for CD1a expression, $CD13^{hi}$ labeling delineates a homogeneous population that should comprise the DC lineage.

*Cytology of day 12 cells.* Cells identified as DCs on cytospins either were large and round in shape with dendritic projections on the surface, abundant cytoplasm and reniform nuclei with dense chromatine and little nucleoli or were smaller with round nuclei but with the same other characteristics (Fig 3A). This pattern could well correspond to R1 and R2 DCs. Most day-12 $CD1a^{+}$ cells had a DC morphology, although CD1a immunostaining was heterogeneous, whereas some $CD1a^{-}$ cells had a DC morphology that was identical to that of the $CD1a^{+}$ cells (Fig 3A). Cells with dendritic morphology were also stained by an LC-specific anti-S100 antibody (Fig 3B).

Although they were $CD14^{+}$, day-12 cells with DC morphology were in fact myeloperoxidase- and nonspecific-esterase negative or weakly positive, whereas other cells, presumably of the macrophage lineage, were strongly positive; only less than 10% of the cells in suspension could phagocytose few latex particles in contrast to adherent cells of the same cultures, the majority of which displayed the macrophage lineage property to ingest numerous particles; the proportion of phagocytic cells increased to 25% on day 20, but these were still poorly phagocytic (data not shown).

*Subsequent evolution of the cultured cells.* This was primarily marked by a decrease of the percentage of $CD1a^{+}$ cells on day 20 relative to day 12 (Fig 4). The phenotype of $CD13^{hi}$ cells was comparable to that of day 12 with respect to CD13, DR, and CD4 labeling, but the CD14 expression level was stronger then (data not shown).

*Characterization of the in vitro DC differentiation pathway.* To more precisely characterize the phenotypic evolution of DCs in vitro, cultured cells were sorted by FACS according to CD13 and/or CD1a expression.

First, sorting of $CD13^{lo}$ versus $CD13^{hi}CD1a^{-}$ cells, excluding those that already expressed CD1a, was performed on day 5. Differentiation of cells of these distinct populations was then examined after further culture. Two days after sort-

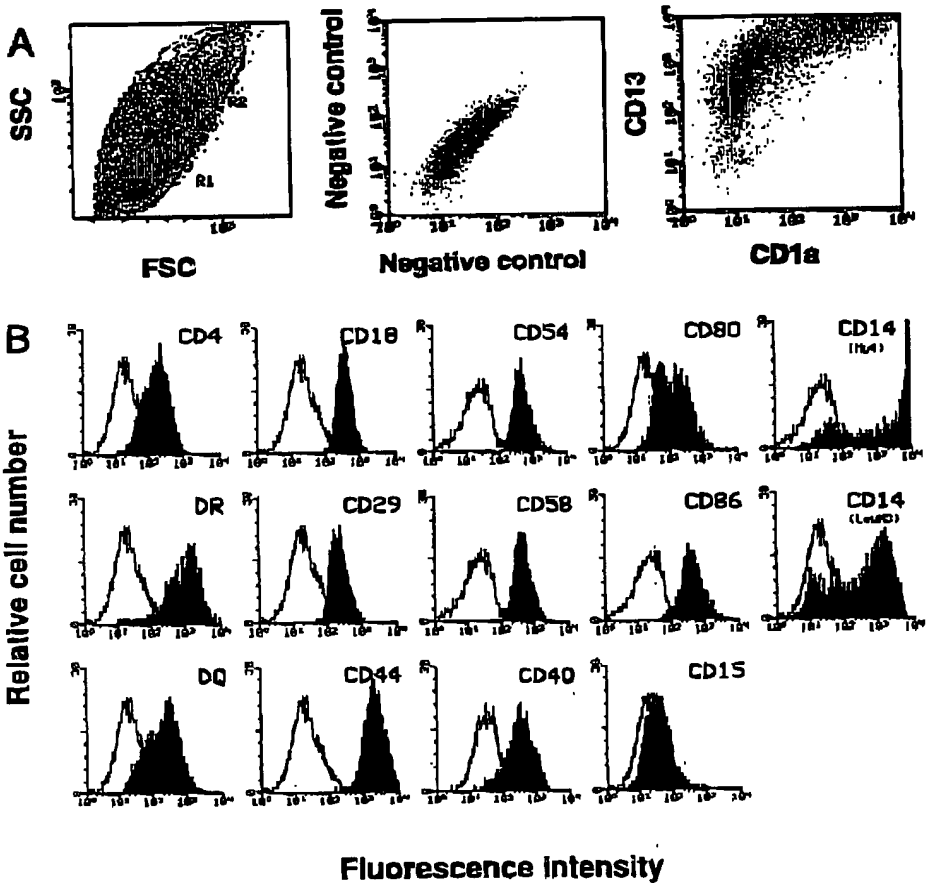


Fig 2. Phenotypic characterization of day-12 cells. Representative light scatter profile of the cells. R1 defines small agranular cells (indicated as red dots) and R2 defines large granulated cells (green dots). Two-color cytograms of the fluorescence intensity of cells labeled either with an isotype-matched irrelevant antibody (control autofluorescence) or with both CD1a-FITC and CD13-PE MoAbs (A). In the latter graph, red and green dots identify R1 and R2 cells, respectively, as determined by counterprojection. FACS analysis of $CD13^{hi}$ cells' expression of CD4; HLA-DR and -DQ; adhesion molecules CD18, CD29, CD44, CD54, and CD58; costimulatory molecules CD40, CD80, and CD86; and myeloid markers CD15 and CD14 (the latter with MoAb My4 or LeuM3) (B). Solid histograms correspond to labeling with the relevant MoAb. Open histograms correspond to negative control labeling as described in the legend of Fig 1.

ing, $CD1a^+$ cells appeared in the initially $CD13^{hi}$ population, whereas $CD13^{lo}$ cells, a minority of which rapidly became $CD1a^+$, emerged from the $CD13^{lo}$ population (Fig 5).

$CD13^{hi}$ cells were next sorted on day 12 according to CD1a expression. Upon sorting, most $CD13^{hi}CD1a^+$ cells had a typical DC morphology (data not shown). They remained $CD1a^+$ and $CD14^+$ in culture, although some $CD1a^-$ cells also appeared, and their number decreased over time. Most sorted $CD13^{hi}CD1a^-$ cells became $CD1a^+$ in culture (Fig 6), with the overall number of cells then remaining stable in culture. Few of them initially had the aspect of DCs, but typical DCs as well as adherent macro-

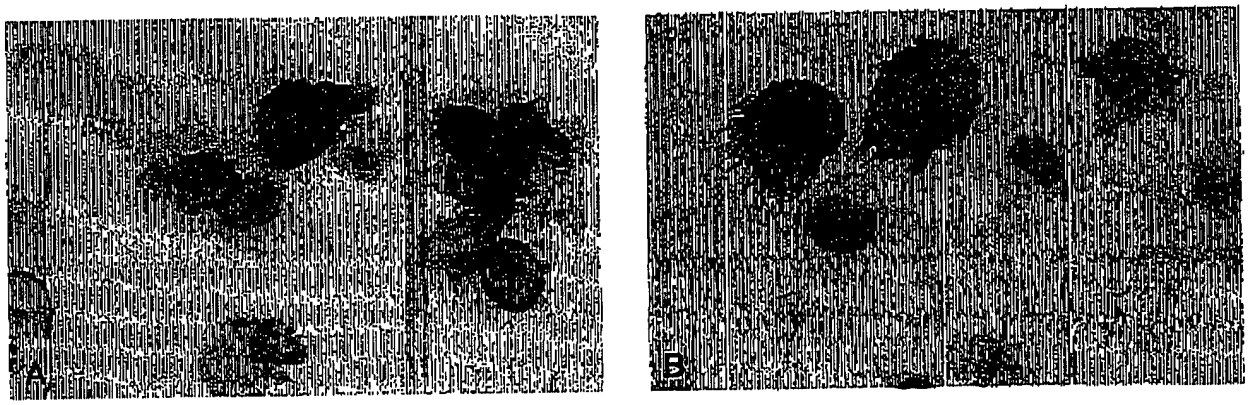


Fig 3. Immunoperoxidase staining of day-12 cells. CD1a (A) and anti-S100 antibody (B) staining of cells with DC morphology.

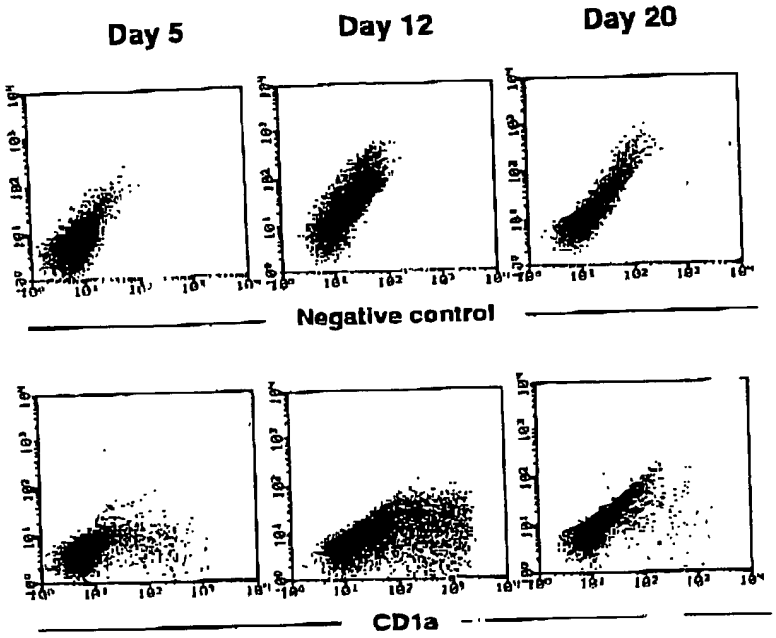


Fig 4. Kinetics of CD1a expression on cultured cells as determined by flow cytometry analysis. Percentages of CD1a$^+$ cells vary here from 5% on day 5 to 40% on day 12 and to 3% on day 20. Negative controls correspond to labeling with an isotype-matched irrelevant antibody.

phages emerged from this population after culture (data not shown).

Altogether, these data suggest the occurrence of a differentiation pathway from CD13$^{lo}$ to CD13$^{hi}$CD1a$^-$ and to CD13$^{hi}$CD1a$^+$ cells. The CD13$^{hi}$CD1a$^-$ cells presumably comprise common precursors to DCs and to macrophages, as already suggested.[3,18,19] It also appears from these results that the CD1a$^+$ cell number decrease noted after 12 days in bulk culture was probably due to the different life span of CD1a$^+$ relative to CD1a$^-$ cells in relation with the culture conditions and perhaps the influence of other cell types, rather than to loss of CD1a expression.

*Effect of the addition of huIL-4 to the cultures after day 12.* To test these hypotheses and because IL-4 has been reported both to inhibit macrophage differentiation and to promote DC maturation from blood precursors,[21,22,29-30] which might be akin to the CD13$^{hi}$CD1a$^-$ cells described here, we examined the effect of adding huIL-4 to cultures starting from day 12.

This led to the appearance of morphologic changes of cultured cells, complete inhibition of adherent cell development, and an increase in the number and size of typical clusters of veiled cells, but without modifying cell numbers (Fig 7).

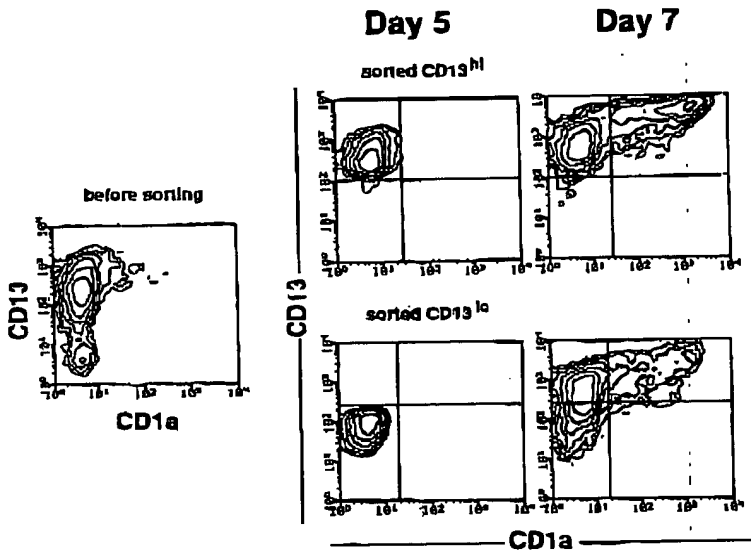


Fig 5. FACS analysis of day-5 sorted cells. After labeling with a CD1a-FITC and a CD13-PE MoAb, cells were sorted according to CD13 expression (CD13$^{hi}$ v CD13$^{lo}$, excluding already CD1a$^+$ cells). This resulted in 95% CD13$^{hi}$ and 94% CD13$^{lo}$ cells. After sorting, cells were further cultured for 2 days and stained with the same MoAbs.

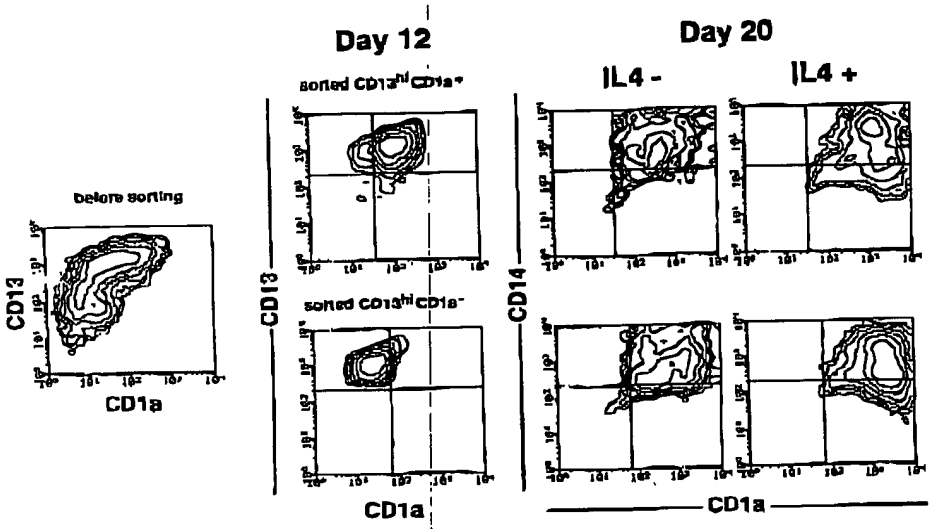


Fig 6. FACS analysis of day-12 sorted cells. Presorted and sorted $CD13^{hi}CD1a^-$ versus $CD13^{hi}CD1a^-$ cells were stained with CD1a-FITC and CD13-PE MoAbs. Sorting resulted in 93% $CD13^{hi}CD1a^-$ and 85% $CD13^{hi}$ $CD1a^+$ cells. CD1a-FITC and CD14-PE labeling of sorted cells cultured for 1 additional week with huGM-CSF + huTNF-α with or without huIL-4.

As shown in Fig 8, which depicts a representative FACS analysis of day-20 cells, the use of huIL-4 led to increase of the "large" cell population on the average from about 50% to 80%; the proportion of $CD1a^-$ cells with greater CD1a and lower CD14 expression levels increased from about 20% to 70%, whereas that of $CD14^+CD1a^-$ cells decreased from 41% to 10%.

When day-12 $CD13^{hi}CD1a^-$ cells sorted from $CD13^{hi}$ $CD1a^+$ cells were further cultured with huIL-4 after 6 days, all cells had become $CD1a^+$ in the presence of huIL-4, compared with about half in its absence (Fig 6). This confirms that day-12 $CD13^{hi}CD1a^-$ cells still contain DC precursors, from which huIL-4 is able to promote DC differentiation, as reported for blood DC precursors.[21,22]

*Capacity of the cultured cells to stimulate the MLR.* To further characterize the cells obtained under different culture conditions in our system, we examined their capacity to stimulate allogeneic adult T lymphocytes in the MLR.

First, day-12 cells cultured with the different combinations of growth factors were irradiated and added in increasing amounts to $5 \times 10^4$ allogeneic blood MNCs. The MLR-stimulating capacity of cells cultured with huGM-CSF and huTNF-α was about 10-fold greater ($P < .05$; two-way analysis of variance) than that of cells cultured with huGM-CSF alone (Fig 9A), as reported,[19,20] whereas cells grown in the additional presence of huSCF for the first 5 days of culture had comparable to fivefold greater MLR-stimulatory capacity ($P < .05$) than when huSCF had not been added (Fig 9B). Also, day-20 cells grown in the presence of huIL-4 had twofold to fivefold greater ($P < .05$) stimulating capacity than without huIL-4 (Fig 9C).

We next determined that the MLR-stimulating capacity of cultured day-12 cells in suspension was about ≥10-fold greater ($P < .01$) than that of the corresponding cord blood adherent macrophages grown under the same conditions (Fig 9D). Nonetheless, comparing day-12 sorted $CD13^{hi}CD1a^+$ versus $CD13^{hi}CD1a^-$ cells showed that, even though both cell types stimulated the MLR, $CD1a^+$ cells elicited the strongest ($P < .05$) response (Fig 9E). These results are consistent with prior findings that DCs have greater MLR-stimulatory capacity than do monocyte/macrophages[4,7,19,20,31] and that CD1a is expressed on more mature DCs that display the strongest antigen-presenting capacity.[12,32]

## DISCUSSION

DCs were initially recognized in the mouse and later in humans.[2] Actually, they represent a broad class of ubiquitous cells,[3,11] which results in a phenotypic diversity, albeit with common morphologic and functional characteristics such as a superior antigen-presenting capacity.[1,3,12] Therefore, the different methods and culture conditions that have been used to obtain DCs, especially from the peripheral blood, do not usually result in fully homogeneous populations, which may account for some of the disparate findings reported as to their characteristics.[7,12,15,16,18,20-22,25,33,34]

Nonetheless, some common phenotypic patterns appear for these cells.[7,12,15,16,18,20-22,25,27,34] They are CD33 myeloid cells that constitutively strongly express major histocompatibility class II HLA-DR, -DP, and -DQ molecules and costimulatory molecules CD40, CD80, and CD86. CD1a expression, which is considered a hallmark DC marker,[2,3,35] seems in fact to be restricted to LCs, to interdigitating cells of lymphoid organs, and to blood DCs that have matured after in vitro culture. There are still discrepancies as regards expression of CD14, CD4, and adhesion molecules.[7,16,33,36,37] Questions remain as to the antigen-presenting capability of these cells according to the differentiation stage,[3,7,38] although it has been shown in mice that maturation of LCs into interdigitating DCs upon migration to lymph nodes corresponds to a process that is associated with reduced antigen-processing capacity and enhanced antigen-presenting function.[39,40]

Different cytokines, mainly GM-CSF but also TNF-α and SCF, play an important role in the maturation of DCs from hematopoietic progenitors.[18-20,25,41] We used here combinations of these cytokines to generate DCs in vitro from puri-

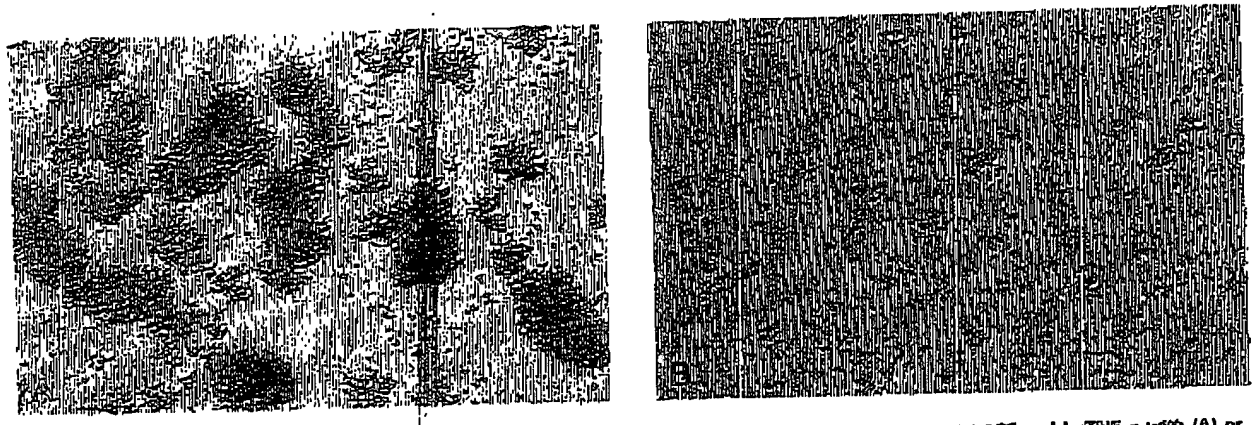

Fig 7. Morphology of day-20 cells. Cultures were conducted from days 12 to 20 in the presence of huGM-CSF and huTNF-α with (A) or without (B) the addition of huIL-4 and were examined by microscopy.

fied cord blood CD34$^+$ precursors. The use of huGM-CSF and huTNF-α[19,20] resulted in the growth and differentiation of cells of the DC lineage, whereas, in contrast to that which is noted with mouse cells,[42,43] neither occurred in cultures with only GM-CSF (data not shown). Adding SCF, which acts on progenitors found at various stages of differentiation,[24,25] to this cytokine mixture during the first days of culture increased overall cell yields without changing the phenotype of the recovered cells or the proportion of DCs.

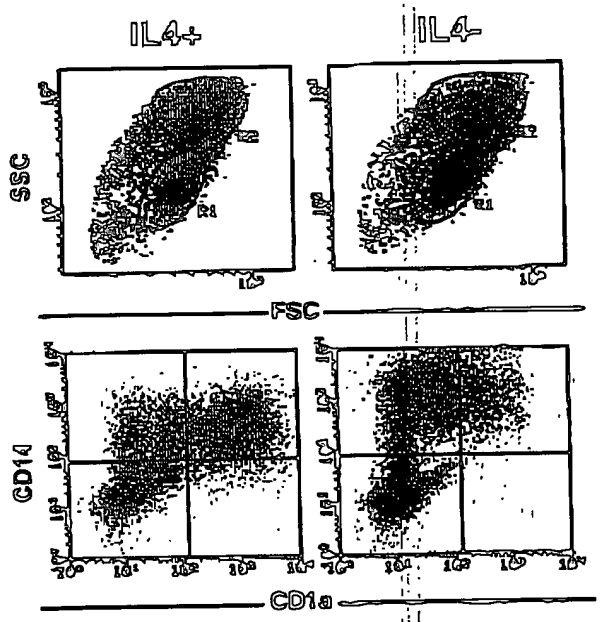


Fig 8. FACS analysis of day-20 cells. Cultures were conducted from days 12 to 20 in the presence of huGM-CSF and huTNF-α with or without huIL-4. The light scatter profile of the cells is shown as well as cell labeling with CD1a-FITC or CD14-PE MoAbs. The percentages of cells in this representative experiment are as follows (culture, with IL-4/without IL-4): %R1, 35/54; %R2, 65/46; %CD14$^+$CD1a$^+$, 50/16; %CD14$^+$CD1a$^-$, 13/42.

A major population of nonadherent mostly large granular cells, characterized by CD13$^{hi}$ expression, was individualized after a few days. CD1a$^+$ cells with characteristic DC morphology continuously emerged from this CD13$^{hi}$ population, at least during the first 2 weeks of culture, at which time they represented 20% to 50% of cells in suspension. At variance with other reports,[20] all CD13$^{hi}$ cells, whether CD1a$^+$ or not, also expressed variable levels of CD14, which is thought to be macrophage lineage-specific,[37] and these levels increased during the third week of culture, whereas the percentage of CD1a$^+$ cells decreased. However, these cells did not express macrophage markers such as nonspecific esterase activity or phagocytosis. Moreover, adding huIL-4 to cultures at that time led to decreased CD14$^+$ and increased CD1a$^+$ cell percentages, with upregulated CD1a and downregulated CD14 expression, which is in line with the fact that DCs can be generated from human peripheral blood precursors cultured with GM-CSF and IL-4.[21,22]

Analysis of the cells' allogeneic T-lymphocyte–stimulating capacity confirmed[4,7,21,31] that DCs stimulated the MLR more than macrophages and that CD1a$^+$ cells, which should correspond to more mature DCs, were the strongest.[13,30,32] The observed differences in MLR activity were smaller than expected, which could be due to the fact that peripheral blood mononuclear cells (PBMCs) instead of purified T cells were used as responder cells. The augmented stimulating capacity of day-20 cells cultured with huIL-4 did not account for the threefold greater proportion of CD1a$^+$ cells noted then. Culturing cells with huSCF for 5 days, without apparently affecting their subsequent phenotype, could increase their MLR-stimulating ability 1 week later. Although none of the investigated adhesion/costimulatory molecules was affected by either growth factor (data not shown), this could indicate that expression of other accessory molecules not examined here was then modified or that the cytokine production pattern of these cells was then different.

Together, these results allow us to delineate a DC differentiation pathway from CD34$^+$ progenitors to CD34$^-$ precursors that is characterized by increased CD13, CD4, and DR expression, from which DCs mature by acquiring CD1a and

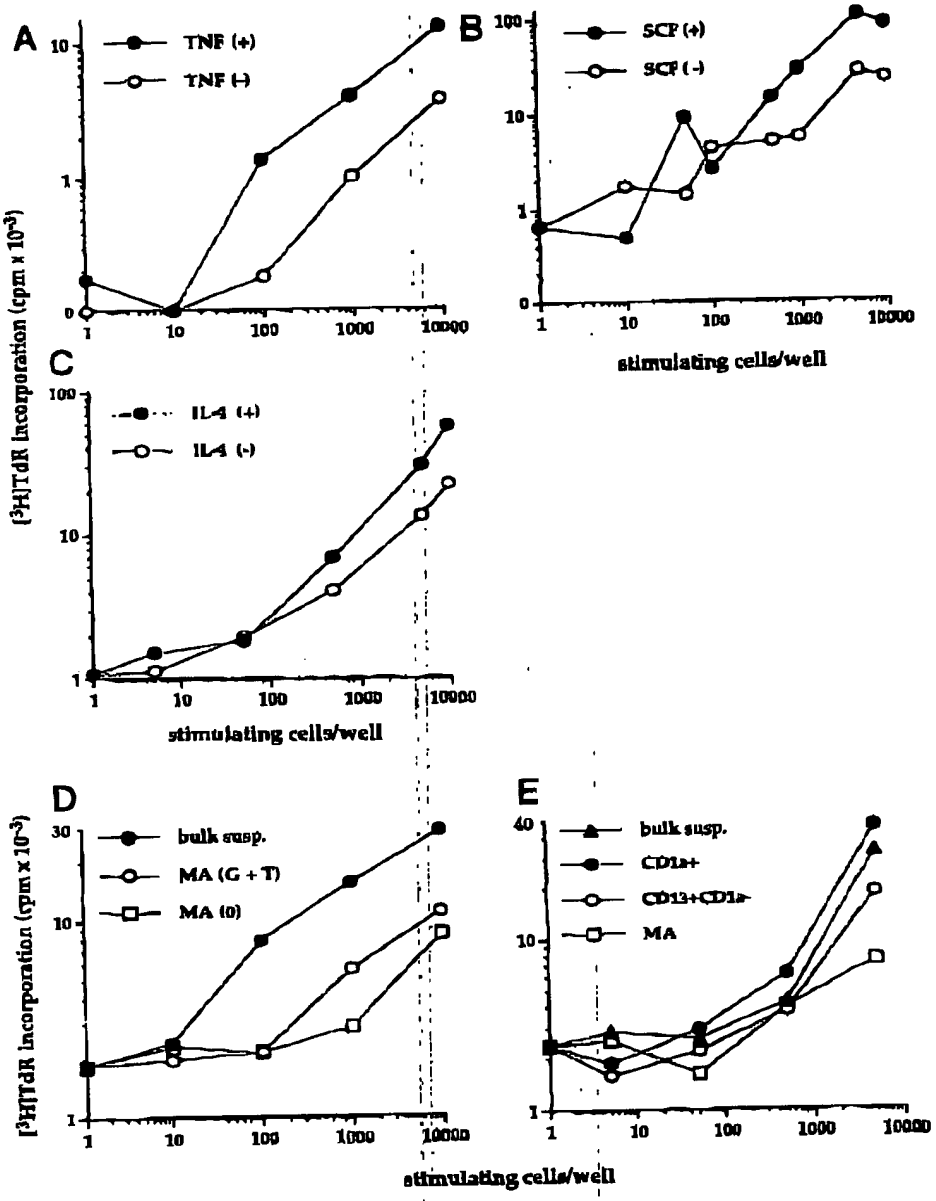


Fig 9. Capacity of the cultured cells to stimulate the MLR. Adult peripheral blood MNCs were cultured for 6 days as responder cells to different concentrations of irradiated stimulator cells that were different populations of the CD34⁺-derived cells: day-12 cells cultured in the presence of GM-CSF, with [TNF (+)] or without [TNF (−)] TNF-α (A) or with [SCF (+)] or without [SCF (−)] SCF for the first 5 days of culture (B); day-20 cells cultured from day 12 onwards with [IL-4 (+)] or without [IL-4 (−)] adding IL-4 to the other cytokines (C). Comparative stimulating capacity of day-12 cultured cells in suspension (bulk susp.) and of cord blood-derived adherent macrophages cultured with [MA (G + T)] or without [MA (0)] GM-CSF and TNF-α (D). Comparison of the stimulating capacity of day-12 CD13$^{bri}$CD1a$^{-}$ (CD1a+), CD13$^{bri}$CD1a$^{-}$ (CD13+ CD1a+) and cord blood macrophages (MA) (E). The data shown are representative of two experiments (A and D), three experiments (B and C), and four experiments (E).

adhesion and costimulatory molecules. Because both typical DCs and macrophages could arise in culture from purified CD13$^{bri}$CD1a$^{-}$ cells, distinct precursors may possibly coexist among this population or CD13$^{bri}$ precursors may rather be common to the DC and macrophage lineages, as suggested by previously reported results of colony assays.[18,19] At any rate, one may consider that DC precursors differentiate in vitro first into CD1a$^{-}$ cells close to peripheral blood DCs and then to CD1a$^{+}$ cells, close to skin LCs, or interdigitating cells of lymphoid organs.[2,3,11,14,15,17,23]

To more precisely define DC differentiation requires using colony and limiting dilution assays, which are currenly under way in our laboratory. Nevertheless, the present results provide the basis to intervene at defined stages of the DC differentiation pathway, and the fact that, in the system developed here, DCs express CD4 from their earlier stage on, makes it a useful tool with which to investigate DC interactions with human immunodeficiency virus.[44-46]

## ACKNOWLEDGMENT

We gratefully acknowledge Micael Yagello's invaluable contribution to cell sorting and FACS analysis experiments. We thank Monique Dutertre. We also thank Ester Remmerswaal for her helpful participation in this work as part of her technician training course in our laboratory. We are indebted to Laurence Boumsell (Laboratoire d'Immunologie, Hôpital Henri Mondor, Créteil, France) for the generous gift of MoAb L544.

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# Isolation of Human Blood Dendritic Cells Using the CMRF-44 Monoclonal Antibody: Implications for Studies on Antigen-Presenting Cell Function and Immunotherapy

By D.B. Fearnley, A.D. McLellan, S.I. Mannering, B.D. Hock, and D.N.J. Hart

**Dendritic cells (DC) are potent antigen-presenting cells (APC) with the capacity to stimulate a primary T lymphocyte immune response and are therefore of interest for potential immunotherapeutic applications. Freshly isolated DC or DC precursors may be preferable for studies of antigen uptake and the potential control of APC costimulator activity. In this report, we report that the monoclonal antibody CMRF-44 can be used to detect early DC differentiation. The majority of DC circulating in blood do not express any known DC lineage specific markers, but can be identified by CMRF-44 labeling after a brief period of in vitro culture. The sequential acquisition of DC activation antigens allows the identification of two stages of DC maturation/activation. Cytokines, especially granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)α, enhance both phases of this process, whereas CD40-ligand trimer preferentially enhances the final DC maturation to a fully mature, activated phenotype. DC positively selected using CMRF-44 possess potent allostimulatory activity and are efficient at the uptake, processing, and presentation of soluble antigens for both primary and secondary immune responses. CMRF-44$^+$ DC are also more potent than other APC types at restimulation of a chronic myeloid leukemia peptide specific T-cell clone. The use of a purified population of freshly isolated DC may be advantageous in attempts to initiate, maintain, and direct immune responses for immunotherapeutic applications.**



DENDRITIC CELLS (DC) are specialist antigen-presenting cells (APC), which play a crucial role in the initiation of a primary immune response. The isolation of DC or their precursors from blood has been difficult in view of their scarcity and the absence of well-established DC lineage markers.[1,2] Alternative means of obtaining DC have been explored and cells with DC-like characteristics have been generated by extended culture of blood, bone marrow, or cord blood precursor cells in the presence of cytokines, typically granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)α or interleukin (IL)-4,[3,4,5] resulting in expansion of cell numbers and differentiation of several lineages, including DC. While many of the cells generated in this manner share functional attributes of DC, these methods do not produce homogeneous populations and there are subtle phenotypic differences between these cells and DC isolated directly from blood or tonsil.[6,7] Attempts have been made recently to clarify the leukocyte populations present and the conditions governing DC ontogeny,[8] although how closely in vitro DC differentiation parallels in vivo events is less clear. It is possible that exposure to self or foreign antigen and the high cytokine levels during extended in vitro culture may result in aberrant DC function.

Immature DC have high antigen processing ability that is diminished on maturation.[9] Therefore, freshly isolated blood DC may be more suitable to the development of immunotherapeutic regimens, as well as providing a suitable DC population for the study of antigen loading mechanisms and regulation of costimulator activity. However, isolation of immature DC from blood by methods involving negative selection do not yield a pure DC population. While expression of HLA class II can be used to identify DC precursors, this approach is not suitable for functional studies because of the effects of anti-class II antibodies on DC function.

The recent development of the CMRF-44[10] and HB15a (CD83)[6] monoclonal antibodies (MoAb), which recognize DC activation antigens, allows positive selection of DC populations from human blood for experimental and possibly clinical immunotherapeutic protocols. We report here that the CMRF-44 antigen is expressed early in the course of DC differentiation from a circulating precursor, and that by observing the differential expression of this antigen and CD83, intermediate stages of early DC differentiation can be identified and some of the factors that influence this process clarified. We were then able to test the hypothesis that the rapid upregulation of the CMRF-44 antigen may be exploited to prepare highly purified DC populations, which retain significant antigen processing activity. We show CMRF-44 purified DC have potent antigen presenting activity after pulsing with synthetic peptide and, therefore, hold promise for use in human immunotherapy protocols. As evidence accumulates that DC not only initiate the immune response, but may also influence the outcome of the T-lymphocyte response,[11] the ability to purify and study DC at different states of differentiation/activation may be useful in manipulating APC function.

## MATERIALS AND METHODS

*Monoclonal antibodies and immunolabeling.* The MoAbs CMRF-15 (anterythrocyte α sialoglycoprotein, IgM), CMRF-31 (anti-CD14, IgG2a), CMRF-44 (IgM), and biotinylated CMRF-44 were produced in this laboratory. HB15a (anti-CD83, IgG2b) was a gift from Dr T. Tedder, Duke University, Durham, NC. The negative controls X63 (IgG1), Sal4 (IgG2b), Sal5 (IgG2a) were a gift from Professor H. Zola (Flinders Medical Center, Adelaide, Australia). Bu63 (CD86, IgG1) was a gift from Dr D. Hardie (University of Birmingham, Birmingham, UK). HuNK-2 (anti-CD16, IgG2a) was a gift from Professor I. McKenzie (Austin Research Institute, Mel-

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*Submitted May 16, 1996; accepted December 30, 1996.*

*Supported by grants from the New Zealand Health Research Council, The Cancer Society of New Zealand, The Canterbury Medical Research Foundation, and Canterbury Health Laboratories.*

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*0006-4971/97/8910-0010$3.00/0*

bourne, Australia). WM54 (anti-CD33, IgG1) and WM15 (anti-CD13, IgG1) were a gift from Dr K. Bradstock (Westmead Hospital, Sydney, Australia). G28-5 (anti-CD40, IgG1), OKT3 (anti-CD3, IgG2a), HNK-1 (anti-CD57, IgM), and OKM1 (anti-CD11b, IgG1) were produced from hybridomas obtained from the American Type Culture Collection (ATCC; Rockville, MD). L307 (CD80, IgG1) and phycoerythrin (PE)-conjugated antibodies to CD14 (leuM3, IgG2b), CD19 (leuM12, IgG1), CD34 (anti-HPCA-2, IgG1), HLA-DR (L243, IgG2a), and PerCP-avidin were purchased from Becton Dickinson (Mountain View, CA). Fluoresecein isothiocyanate-conjugated sheep antimouse immunoglobulin (FITC-SAM) was purchased from Silenus (Hawthorn, Australia).

Labeling was performed by standard techniques. Briefly, cells were incubated with saturating concentrations of primary antibody for 30 minutes at 4°C, washed twice, incubated with FITC-SAM for 30 minutes at 4°C, washed twice, blocked with 10% mouse serum for 5 minutes and then incubated with PE-conjugated or biotinylated second antibody. For biotinylated antibodies, a further washing step was followed by incubation with PE or PerCP-avidin for 30 minutes and a final washing before analysis or sorting on a fluorescence-activated cell sorter (FACS) Vantage (Becton Dickinson, Mountain View, CA). Samples that could not be analyzed immediately were fixed in 1% paraformaldehyde and stored at 4°C.

*Cell preparation.* Blood was obtained from volunteer donors with appropriate informed consent according to Ethical Committee guidelines. Peripheral blood mononuclear cells (PBMC) were prepared by isolation over sterile Ficoll/Hypaque (d = 1.077 g/mL; Pharmacia, Uppsala, Sweden) gradients. T lymphocytes for functional assays were prepared from PBMC by rosetting with neuraminidase-treated sheep erythrocytes, followed by Ficoll/Hypaque separation and erythrocyte lysis with distilled water.

*Dendritic cell preparation.* (1) For experiments involving phenotypic analysis of freshly isolated and cultured DC, freshly isolated PBMC were depleted of T lymphocytes as above and then labeled with a mix of CD3, CD11b, CD14, CD16, and CD19 MoAb. After incubation with goat antimouse Ig-coated magnetic microspheres (Miltenyi Biotech, Germany), labeled cells were removed by magnetic immunodepletion and the MoAb negative cells were then labeled with FITC-SAM and further purified by sorting using a FACS Vantage flow cytometer, if required. Two or three color immunofluorescent labeling was then used to identify DC on the basis of HLA-DR and/or CMRF-44 staining.

(2) For functional studies, DC were purified by positive selection using CMRF-44 labeling. Briefly, PB non-T cells were isolated as above and then cultured at $2 \times 10^7$/mL for 12 to 15 hours. Low density cells were then isolated by separation over a Nycodenz (Nycomed Pharma, Norway) gradient (d = 1.068 g/cm³) as previously described.[12] Cells were washed three times and labeled sequentially with CMRF-44, FITC-SAM, and CD14-PE. CMRF-44$^{++}$, CD14$^-$ cells were sorted to high purity and used for functional studies.

*Cell lines.* The HLA-DR1–restricted CD4 T-lymphocyte line (NG-1), which recognizes a chronic myeloid leukemia (CML)-specific peptide derived from the b3a2, bcr-abl fusion protein arising as a consequence of the 9:22 translocation was generated in this laboratory.[13] NG-1 was maintained by periodic restimulation with b3a2 peptide plus autologous MNC feeder cells and was rested before use in antigen presentation experiments.

*Medium and cytokines.* Cell culture medium used unless otherwise stated was RPMI-1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 10% heat inactivated fetal calf serum (FCS) (Life Technologies, Auckland, New Zealand). AIM-V medium (Life Technologies) was used for experiments performed in serum-free conditions. In some experiments, base media was supplemented with one of the following cytokines: IL-2 (100 U/mL), TNFα (20 ng/mL; both from Hoffman-La Roche, Basel, Switzerland), IL-3 (20 ng/mL; R & D Systems, Oxford, UK), IFNγ (500 U/mL; Boehringer-Ingelheim, Ingelheim, Germany), IL-4 (25 ng/mL; Sigma, St Louis, MO), or GM-CSF (500 U/mL; Sandoz, Basel, Switzerland). Murine CD40-ligand trimer (mCD40-LT), which is capable of binding human CD40 (gift from Dr M. Widmer, Immunex, Seattle, WA) was used at 10 μg/mL.

*Functional assays.* Allogeneic mixed lymphocyte reaction (MLR): $10^5$ T lymphocytes were cultured at 37°C in 5% $CO_2$ in 96-well plates with triplicate graduated numbers of sorted APC subsets obtained from a single allogeneic donor. Wells were pulsed for 12 hours with 0.5 μCi tritiated thymidine (Amersham International, Arlington Heights, IL) immediately before harvest at 5 days. Cells were harvested onto filter paper and thymidine incorporation was measured with a liquid scintillation counter. Data are expressed as mean counts per minute (CPM) of triplicate wells ± standard deviation (SD). Control wells containing T cells or APC alone incorporated <500 cpm of tritiated thymidine in all experiments.

Soluble protein or peptide presentation assays and autologous MLR: These assays were performed following previously published methods[14,15] with minor modifications. Briefly, $10^5$ T lymphocytes were cultured with 5,000 autologous APC without antigen or with 10 ng/mL keyhole limpet hemocyanin (KLH; Sigma) or 1 μg/mL tetanus toxoid (Commonwealth Serum Laboratory, Melbourne, Australia) in 96-well plates. Each well was harvested after 8 days following a 12-hour pulse with 0.5 μCi tritiated thymidine. Results are expressed as mean CPM of triplicate wells ± SD. For assays of peptide presentation, $2.5 \times 10^4$ NG-1 responder T lymphocytes were cultured with graduated doses of peptide pulsed (10 μg/mL) APC obtained from HLA-DRB1*0101 donors, and thymidine incorporation was assayed after 3 days.

## RESULTS

*A CMRF-44 bright, putative DC population can be identified after in vitro culture of freshly isolated PBMC.* We have previously established that CMRF-44 labels B lymphocytes, monocytes, and DC, but does not stain other blood cells.[10] We now extend this data to show that a strongly CMRF-44 positive, putative DC population is discriminated from CD19$^+$ B lymphocytes and CD14$^+$ monocytes by the intensity of CMRF-44 staining. Enumeration of the CMRF-44$^{++}$ (bright) population after 24 hours of culture estimated the DC percentage at 0.2% to 1% of PBMC (n = 7). This percentage increased to 2% to 3% (n = 3) at 48 hours (Fig 1A). The CMRF 44$^{++}$ (bright) population also express higher levels of HLA-DR than other cultured PBMC (Fig 1A). The relatively DC-specific CD83 antigen[6] is recognized on a similarly sized population by HB15a labeling (Fig 1B). Ongoing maturation of precursors, further DC division, or preferential survival of DC probably accounts for the increase of CMRF-44 bright cells. The relative contribution and kinetics of these processes varied between individuals and may account for the variation in DC yields seen between individual donors. The CMRF-44 bright (non-B lymphocyte/monocyte population) was also generated in commercially prepared, (endotoxin-free) serum-free media (AIM V) (n = 5). Again, considerable interindividual variation in DC numbers was observed between donors, making it unlikely that this variability is solely due to idiosyncratic reactions to lipopolysaccharide (LPS) or similar substances that may be present in serum.

*Small numbers of CMRF-44 positive DC are present in*

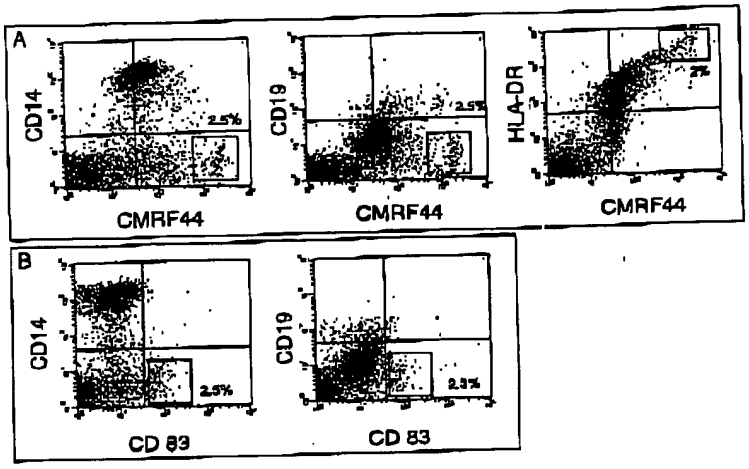


Fig 1. CMRF-44 labels cultured peripheral blood DC. T-lymphocyte depleted PBMC were cultured for 48 hours and labeled with the MoAb indicated. The quadrants were placed according to isotype matched control staining performed at each time point. The percentage of cells with (A) the CMRF-44 bright or (B) HB15a$^-$ phenotype (shown in rectangle) was 2% to 2.5% in this individual. Antibody labeling intensity is displayed across 4 decades of log fluorescence in all figures. Results are from one of three similar experiments.

*freshly isolated PBMC and DC rapidly upregulate CMRF-44 during in vitro culture.* To examine the upregulation of the CMRF-44 and CD83 antigens on the putative blood DC precursor population, we prepared PBMC and depleted mature leukocyte populations as described in the Materials and Methods section. The resultant lineage negative population (typically < 4% of freshly isolated PBMC) included 30% to 80% HLA-DR positive cells. CMRF-44 labeled the most strongly HLA-DR staining cells before in vitro culture and subsequently labeled 50% to 80% of the HLA-DR positive cells after a short period of culture in serum containing medium (Fig 2A). The CD83 antigen was not expressed on these freshly isolated DC precursor populations initially, but was detected after culture on the HLA-DR bright cells (Fig 2A). The expression of the CMRF-44 antigen preceded that of the CD83 antigen (Fig 2B) at early time points, although beyond 24 hours, the percentage of cells labeled by CMRF-44 and HB15a was essentially the same (Fig 1A and B).

Three distinct phenotypic states of activation/maturation can be clearly identified in conjunction with changes in cell size. Progression from an HLA-DR positive, CMRF-44$^{-/+}$ CD83$^-$ DC precursor population through an HLA-DR positive, CMRF-44$^+$ CD83$^{-/+}$ intermediate state to an HLA-DR bright CD83$^+$ and CMRF-44$^{++}$ mature DC was observed (Fig 2A). The HLA-DR$^+$ lineage mix negative population possess the forward and side scatter characteristics of lymphocytes, the intermediate stage of CMRF-44$^+$ cells fall between the lymphoid and monocytoid gates, and the CMRF-44$^{++}$ cells are found in the monocyte gate (data not shown). The phenotype of these populations was analyzed and is summarized in Table 1 (data obtained from a minimum of three experiments per antigen). Of note, the CD40, CD80, and CD86 antigens increase in density on DC as they progress through these steps.

In the majority of experiments, an almost linear relationship between HLA-DR and CMRF-44 labeling was seen. It should be noted that the CMRF-44 antigen has been characterized as a glycolipid and distinguished from the currently known HLA-DR products[11] and that CMRF-44 does not label HLA-DQ or DR-transfected L cells, or the small number of CD34 positive cells (known to be DR and DP positive) also present in the lineage negative, HLA-DR positive population.

*The short-term development of DC can be augmented with additional cytokines.* When PBMC preparations are cultured (as above), DC development may be supported by cytokines or other products released by mature cells, as well as serum additives in the culture media. The effect of cytokine supplementation on early DC differentiation was examined in the absence of mature leukocytes after freshly isolated, lineage mix negative cells (prepared as described earlier) had been cultured for 40 hours in the presence of additional cytokines. In contrast to the results seen in above, there was less DC differentiation and lower DC viability in serum-free conditions indicating that these lineage mix negative cells alone are either unable or not sufficiently stimulated to produce cytokines needed for this process. The addition of 10% FCS had a greater effect than any individual cytokine or TNF$\alpha$, GM-CSF, and IL-4 combined (not shown).

In the presence of 10% FCS, GM-CSF, and TNF$\alpha$, both increased cell viability and consistently enhanced early maturation from early and intermediate DC precursors, as judged by both absolute CMRF-44 positive cell numbers and mean fluorescence intensity (MFI) of CMRF-44 labeling (Fig 3). The increase in CMRF-44 expression induced by additional cytokines (relative to that induced in RPMI/10% FCS alone) was 135% of control with TNF$\alpha$ (95% confidence interval [CI], 110% to 155%) and 128% of control with GM-CSF (95% CI, 98% to 173%). IL-3 and IFN$\gamma$ had a similar, but less marked effect on increasing the number of CMRF-44 and CD83 positive cells in some experiments. The presence of CD40-LT did not enhance the maturation from early DC precursors, but did increase the MFI of HLA-DR, CMRF-44 (Fig 3), and HB15a labeling (not shown) on the more mature DC.

In all experiments, a variably sized population of HLA-DR positive cells was present, which did not acquire the CMRF-44 positive phenotype of DC during the culture period (Figs 2 and 3). There was no evidence that these cells

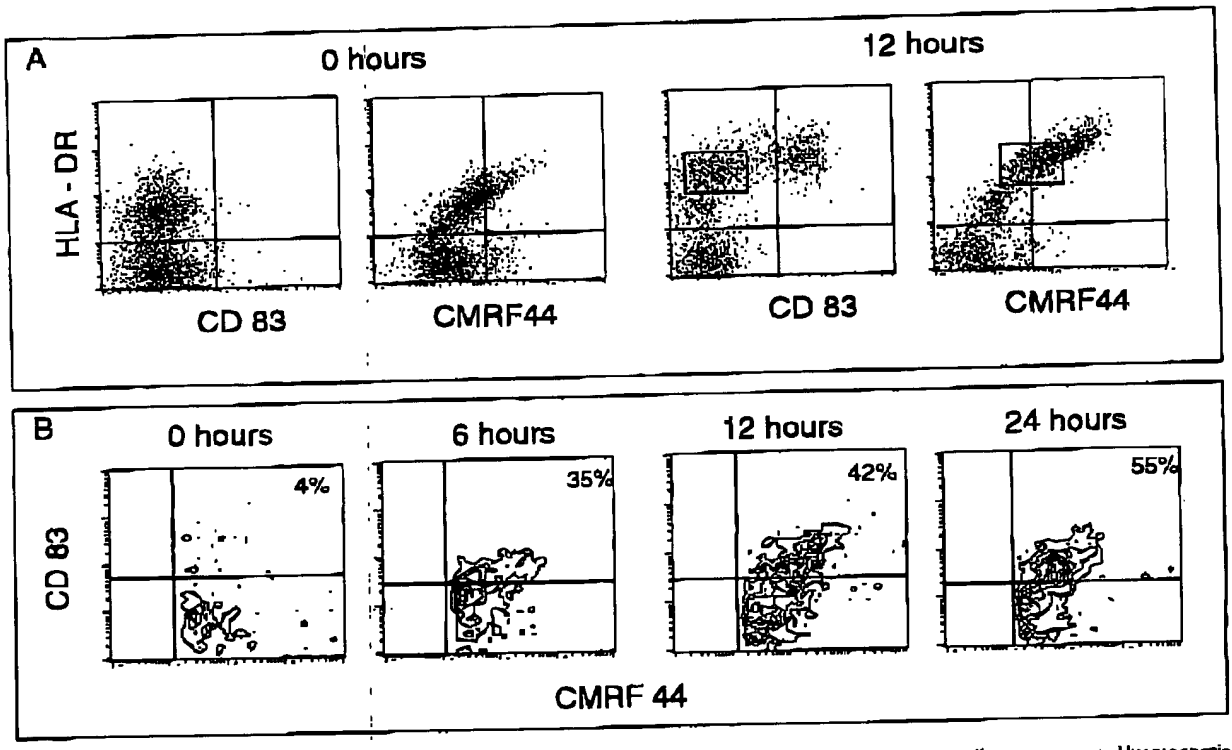


Fig 2. CMRF-44 positive blood DC can be detected before culture. (A) Mature lineage positive mononuclear cells were removed by magnetic immunodepletion. The resulting lineage negative cells were labeled before (0 hours) and after 12 hours culture. A lineage negative, CMRF-44 positive subpopulation is noted. The differential expression of the activation antigens recognized by CMRF-44, HB15a (CD83), and anti-HLA-DR antibodies allow the identification of distinct stages of early DC development (see text). The rectangle indicates the CMRF44+/CD83− stage of DC differentiation . (B) Time course labeling of cultured lineage negative cells gated on CMRF-44 positive cells showing the differential expression of the CMRF-44 and CD83 antigens. No CD83+/CMRF-44− cells were detected at any time point. Quadrants are placed according to isotype-matched negative controls such that <95% of events are excluded from the right-hand quadrants. Results are from one of three similar experiments.

could differentiate into CD14 positive monocytes under these conditions (not shown). The phenotypic data (Table 1) suggests that the HLA-DR positive, non-DC, population comprises a mixture of CD34+ precursors, other immature myeloid cells (CD13 and/or CD33 positive), activated T lymphocytes (CD3 low, CD4+,CD25+), and possibly other cell types. A variable percentage of HLA-DR negative cells are present in lineage negative PBMC preparations, but they did

Table 1. Phenotype of the Lineage Mix Negative, HLA-DR Positive Cells (Before and After Culture) and CMRF-44++ Sorted DC

| Leukocyte Differentiation Antigen | Lineage Mix Negative Cells | | | | CMRF-44+ Sorted DC (see Fig 4) |
|---|---|---|---|---|---|
| | | Cultured (phenotypic subpopulations) | | | |
| | Fresh* HLA-DR+ CMRF-44(+) | *HLA-DR+ CMRF-44− | HLA-DR+* CMRF-44+ | HLA-DR−− CMRF-44−− | HLA-DR++ CMRF-44++ |
| CD83 | − | − | − | + | + |
| CD40 | − | − | + | ++ | ++ |
| CD80 | − | − | (+) | + | + |
| CD86 | − | − | + | ++ | ++ |
| CD54 | + | + | + | ++ | ++ |
| CD11b | (+) | (+) | − | + | + |
| CD13 | (++) | (−/+) | + | + | + |
| CD14 | − | − | − | − | − |
| CD33 | (+) | − | + | + | + |
| CD4 | (+) | (+) | (−/+) | + | + |
| CD25 | − | (+) | − | + | + |

Abbreviations: −, negative; −/+, weakly positive; +, positive; ++, strongly positive; ( ), subpopulation only.

* Denotes heterogeneous population.

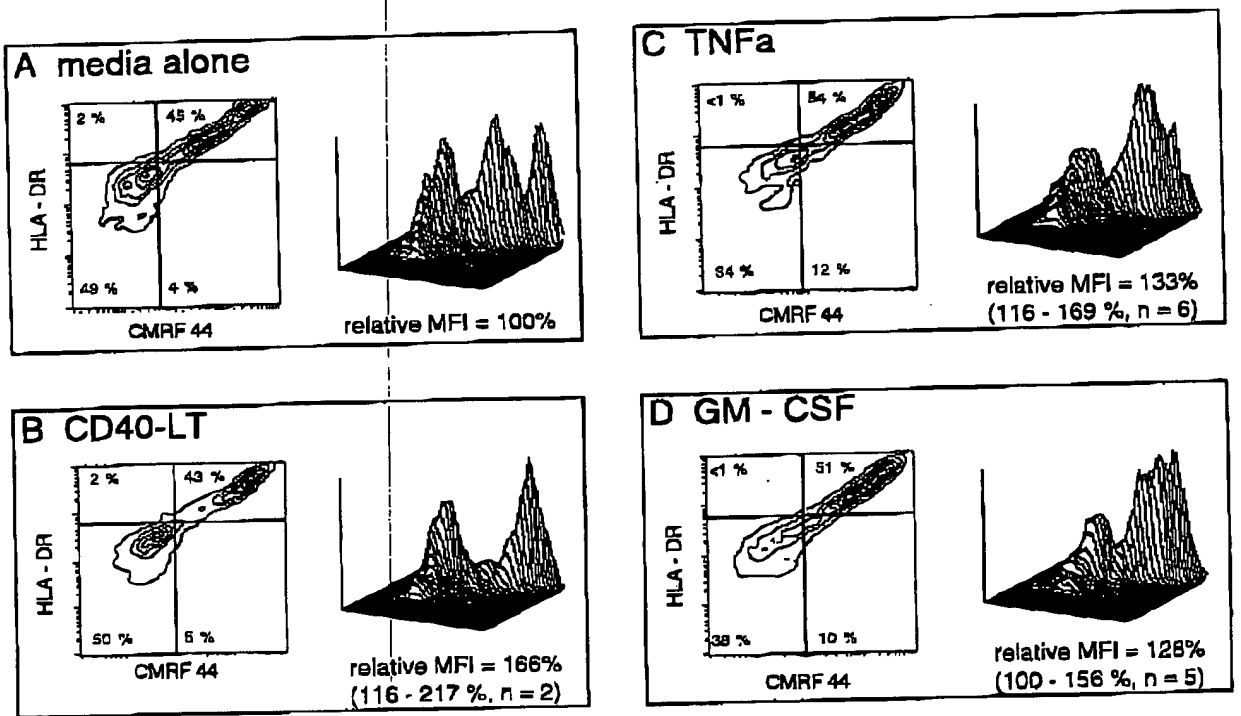


Fig 3. Short-term development of CMRF-44$^{++}$ DC in culture is augmented by additional signals. Freshly isolated PB lineage marker negative cells were prepared (as described in Materials and Methods) and were cultured for 40 hours in media alone or with additional cytokines as shown. The plots are gated on live cells (as assessed by forward and 90° light scatter characteristics) which expressed HLA-DR. A total of 10,000 events were collected in each case using identical gating criteria. The relative mean fluorescence intensity (MFI) of CMRF-44 labeling in each case was calculated using the formula: MFI CMRF-44 (media + cytokine) - MFI (control Ab)/MFI CMRF-44 (media alone) - MFI (control Ab) and expressed as a percentage. Additional GM-CSF or TNFα both increased the number of viable cells by up to 20% compared with media alone. Quadrants are placed as dictated by isotype matched control antibody (x axis) and at the level of class II expressed on these cells before culture (y axis). Percentages of cells falling into each quadrant are shown. The three-dimensional plots depict the same data and allows a comparison of the cytokine effect at each stage of DC development.

not label with either CMRF-44 or HB15a under any culture conditions, although IFNγ induced HLA-DR in a small percentage of these cells.

*The rapid induction of CMRF-44 can be exploited to simplify the purification of DC.* Previous experiments[12] have shown that during short-term (12 to 16 hours) in vitro culture of T-lymphocyte–depleted PBMC, DC reduce their buoyant density. Isolation of low density cells over a Nycodenz gradi-

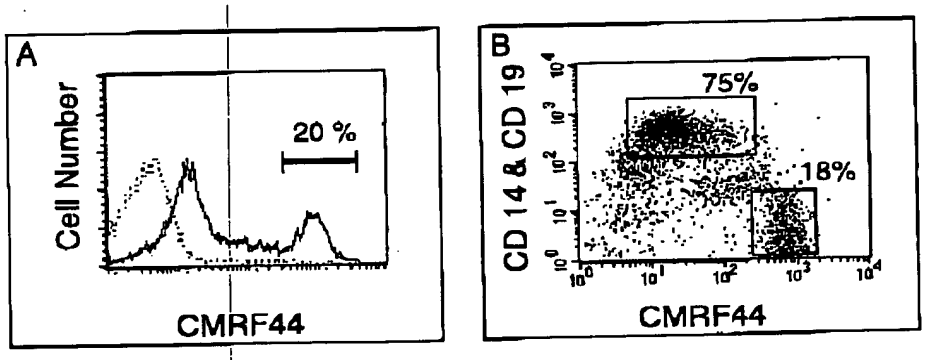


Fig 4. CMRF-44 provides a convenient method for blood DC purification. (A) After in vitro culture of peripheral blood ER negative cells, density gradient separation typically enriched CMRF-44 bright DC population from <1% to 15% to 25%. (B) Double labeling with CD14 to identify the coenriched low density monocytes (70% to 80% of low density cells) allows clear separation of the CMRF-44 bright population (15% to 20% of low density cells), which can then be sorted to high purity. In this example, the CD19-PE has been added to show the small (1% to 2%) population of B lymphocytes also present. Typical sort regions are shown.

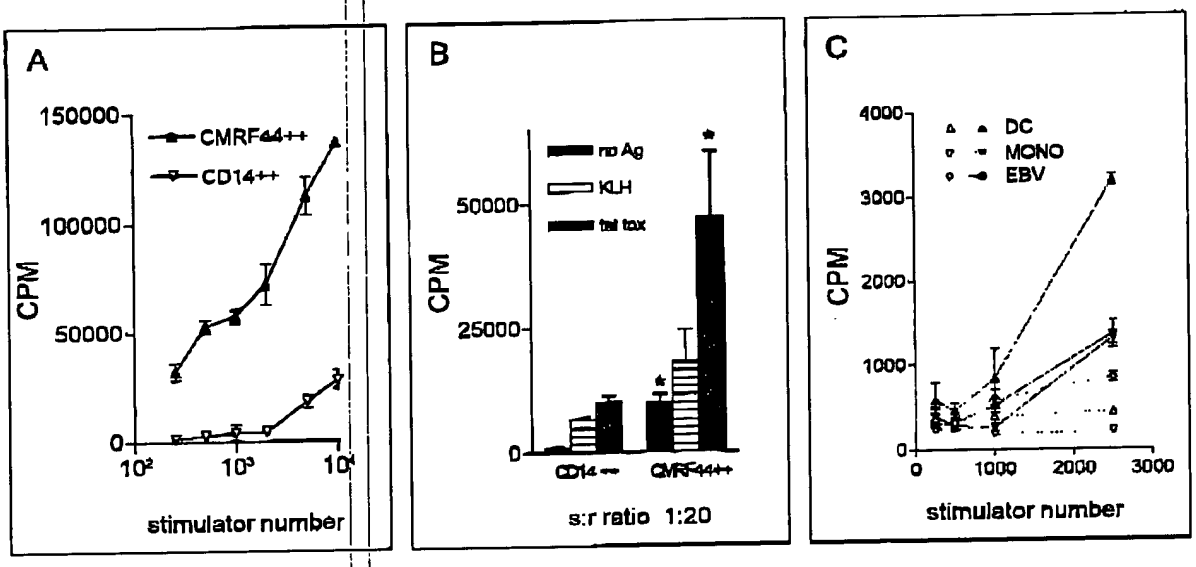


**Fig 5. CMRF-44 positive cells have potent antigen presenting capacity. CMRF-44 bright CD14 negative DC were compared with CD14 bright CMRF-44 dim monocytes in their ability to (A) stimulate an allogeneic MLR, (B) process and present primary and recall antigens to autologous T cells (* represents significant ($P < .05$ by student's $t$ test) difference between DC and monocytes), (C) or present peptide antigen to an established CML fusion protein bcr-abl specific T-cell line (open symbols, no antigen; closed symbols, b3a2 peptide). In all cases, CMRF-44 bright DC show greater stimulatory activity than other APC types, emphasizing the potential of these cells for immunotherapeutic applications.**

ent enriched the CMRF-44 bright cells from <1% of the starting population to 15% to 25% of the low density fraction (Fig 4A). The expression of CMRF-44 on monocytes varies between individuals, therefore double labeling with CD14 to identify the coenriched low density monocytes was used to ensure that the CMRF-44 bright, CD14 negative population was sorted to high purity. Typical two color labeling and sort regions for DC ($CMRF\text{-}44^{++}$, $CD14^-$) and copurified, low density monocytes ($CMRF\text{-}44^{-/+}$, $CD14^+$) are shown (Fig 4B) as representative of over 25 experiments. The viability of the sorted cells was typically >85%, reducing to ≈ 50% after a further 24-hour culture, as expected when the sorted DC are cultured in isolation. Morphologically, the sorted DC show some heterogeneity, probably as a result of differential maturation status, but the majority are medium/large cells with a characteristic irregular nucleus. The phenotype of the sorted cells is shown in Table 1. The CMRF-44 antibody does not affect the allostimulatory potential of DC,[11] but as yet, no further functional data on the antigen is available.

Using this method 0.5 to 2 × $10^5$ DC can be obtained from 100 mL blood. The technique can be readily scaled up to handle larger cell numbers, such as an apheresis product, from which sufficient DC (1 to 10 × $10^6$) for clinical applications may be obtained.

*Low density, CMRF-44 positively selected cells possess the functional attributes of DC.* Sorted CMRF-44 bright DC were potent stimulators of the allogeneic MLR with as few as 200 DC showing significant activity. The purified DC were 10 to 100 times more stimulatory than the ($CMRF\text{-}44^{-/+}$, $CD14^+$) monocytes, which were also isolated from the Nycodenz gradient (Fig 5A, representative of five experiments).

A critical observation was that the CMRF-44 bright cells retain the ability to process and present antigen to autologous T lymphocytes after a 12-hour period of in vitro culture. When CMRF-44 bright cells were cultured with autologous T lymphocytes, a substantial background autologous MLR response was generated. Pulsing the cells with KLH resulted in additional T-lymphocyte proliferation, establishing that these CMRF-44 bright cells can take up and process antigen effectively and present the resulting KLH peptides to generate a primary T lymphocyte response (Fig 5B, representative of three experiments). A similar specific and more substantial secondary T-lymphocyte response to tetanus toxoid was also seen (Fig 5B). It is noteworthy that high DC stimulator ratios generated increasing nonspecific responses, suggesting lower DC:T lymphocyte ratios are better for in vitro initiation of a specific response.

Finally, CMRF-44 bright cells are also capable of presenting a synthetic CML bcr-abl fusion protein antigenic peptide to a T-cell line specific for this peptide (Fig 5C, representative of two experiments on HLA-matched donors). An effective response occurred even at very low stimulator to responder ratios and it was clear that DC showed greater stimulatory activity for this particular secondary T-lymphocyte response than other APC types tested.

## DISCUSSION

Blood DC arise from an HLA class II positive, lymphoid sized cell that lacks distinguishing morphological or phenotypic features. These experiments establish that the CMRF-

44 antigen is an early distinctive marker of DC maturation and that the majority of CMRF-44 positive cells subsequently coexpress the CD83 antigen. Maturation of murine DC is accompanied by a burst of HLA class II and invariant chain synthesis[16] and a similar striking upregulation of HLA class II antigens is seen in these experiments on differentiating human blood DC, intimately accompanied by upregulation of the CMRF-44 antigen. Constitutive and inducible HLA class II expression are probably regulated by different mechanisms[17] and the parallel expression of HLA class II and CMRF-44 on DC raises the possibility that CMRF-44 expression may be under similar control as inducible HLA class II. The fact that the gene encoding murine CD83 has been localized to the major histocompatability complex (MHC) region recently[18] raises the intriguing possibility that it too may be regulated by similar mechanisms.

The HLA class II positive, lineage negative population found in fresh blood is heterogeneous and includes circulating $CD34^+$ stem cells, blood DC and immature forms of other cell lineages. Under the conditions used in these experiments, it appears that the majority of HLA class II positive, lineage negative cells can acquire the phenotype of DC, although the rate at which this occurs varies between individuals. Monitoring the cells that remain HLA class II positive, CMRF-44/CD83 negative after culture indicates a mixed population of cells, with no evidence of B lymphoid or monocytic commitment. Conceivably antigen loss during the immunoselection involved in purification[19] may contribute to this population. It is also possible that some of these cells may represent either less mature cells, which require more time to express lineage-specific antigens, or may be a cell population replenished by cell division. The presence of a heterogeneous subpopulation of HLA class II negative cells is also acknowledged, however, whether any of these populations significantly affect DC differentiation remains unclear. The existence of the CMRF $44^+/CD83^-$, intermediate stage of DC differentiation identified in these experiments is supported by the phenotypic similarities these cells have with mature DC (Table 1), and their expression of CD83 after longer culture periods or in the presence of additional cytokines. This phenotype may be useful in identifying tissue DC, which have been partially activated in vivo.

In vivo, maturation of DC precursors has been considered to occur in two stages. Initial changes occur in tissues and further activation/differentiation to a fully mature/activated phenotype typically occurs on migration to a lymph node. In these in vitro experiments, a similar, biphasic maturation was seen over a relatively short time period. Both TNF$\alpha$ and GM-CSF can initiate this process in vitro[20] (as can other cytokines to a lesser extent – perhaps via indirect effects), but whether these signals are prerequisites for in vivo DC lineage commitment is still unclear. The identification of a small $CMRF\text{-}44^+$, maturing DC population in blood is in keeping with other reports of a similar population[21] and suggests there is a basal level of DC generation from precursors, which can be substantially augmented in the presence of inflammatory cytokines. The observation that 10% FCS supported DC maturation better than a combination of GM-CSF, TNF$\alpha$, and IL4, when added to serum-free media, suggests that other factors may be important to in vivo DC maturation, as does the preservation of normal DC production seen in GM-CSF gene-targeted knockout mice.[22]

The blood DC, which appear to have undergone a degree of activation, may have been activated via interactions with T lymphocytes or endothelial cells, although it is possible that even the minimal cell handling before culture might induce CMRF-44 expression. Increased surface expression of the CD40, CD80, and CD86[23,24] molecules, which contribute significantly to DC costimulation, accompanies the CMRF-44 upregulation during DC activation/maturation. It is interesting to note that CD25 (IL-2R$\alpha$) expression also upregulates on DC during this process. The absence of a fully activated CMRF-44 bright DC population may argue partly against the existence of a recirculating population of activated blood DC and the two activation states of DC we have documented in freshly isolated cells might explain the apparent heterogeneity of blood DC observed by others.[25]

Uptake and processing of antigen has been reported to be most efficient in immature DC[5,9] and much of the antigen processing ability of cytokine cultured murine DC has recently been shown to be attributable to the persistence of immature cells.[26] MHC-DR molecules synthesized during activation are more stable and remain on the surface of DC for longer than those expressed constitutively.[16] Pulsing blood DC with antigen during this process of HLA class II synthesis may optimize antigen DC loading for therapeutic purposes. It is significant, therefore, that the mechanisms of DC antigen uptake appear to be active in CMRF-44 purified DC, as supported by the ability of the 12-hour cultured DC to process and present the soluble antigen KLH in a primary response and both protein (TT) and peptide (bcr-abl) antigens in secondary responses.

A potential T lymphocyte effect on late DC maturation mediated via CD40L/CD40 was noted here, further emphasized by the CD40L/CD40-mediated enhancement of blood DC costimulator molecule expression seen previously.[23,27] Full DC differentiation and activation of costimulator activity probably requires interaction with T lymphocytes and this may impart a degree of antigen specificity to DC activation. This is interesting in view of recent suggestions that the signals that activate DC influence whether tolerance or immunity result from a DC-T cell interaction.[28,29] While the viability of our sorted DC was higher than that previously reported,[30] these rather low survival figures may provide a second line of evidence for activated DC dependence on interactions with other cells, particularly antigen-specific encounters with T lymphocytes, to attain a state of full maturation.

DC appear the logical APC for immunotherapeutic applications, providing sufficient cells can be obtained via clinically acceptable protocols. Efficient antigen loading into purified DC may enable the generation of T responses against some antigens, which otherwise would not provoke an in vivo response. Cell preparations containing DC obtained from precursors cultured in cytokine mixtures are efficient APC and can be generated in relatively large numbers, however, the use of these heterogeneous populations in a clinical setting raises several concerns. First, the extended period of

in vitro culture maximizes exposure to auto antigens. Second, the high cytokine concentrations used in these cultures may result in aberrant cell function, including possible resistance to normal regulatory mechanisms. Third, the contaminating cells may induce counterproductive outcomes, either by producing cytokines such as IL-10 or TGF$\beta$ or by altering the balance of costimulatory signals, thereby influencing whether or not T lymphocytes are activated efficiently following T-cell receptor (TCR) engagement[31] or modifying the cytokine profile of responding T lymphocytes.[32] Moreover, the methods used to produce cytokine-generated DC vary, and both the cell types generated and their relative number may differ between laboratories.

The use of blood DC purified without a prolonged in vitro culture period or additional cytokines might simplify attempts to use DC in a clinical setting. Furthermore, manipulation of DC to direct T lymphocyte responses in vitro would be best addressed using purified DC. These purified populations can also be used for studying DC antigen uptake, processing, and control of costimulator function at the cellular or molecular level. Variables to be considered (apart from APC numbers) include the dose of antigen,[33] the form of antigen processed by the DC,[34] and the possibility of activation-induced T lymphocyte or DC death.[35] Given the potency of DC, large numbers of DC may not be necessary to successfully generate an in vivo T-lymphocyte response and the efficacy and safety of relatively small numbers of freshly isolated DC has already been demonstrated in humans.[36]

## ACKNOWLEDGMENT

We are grateful to Aaron Rae and Lisa Whyte for expert FACS assistance and Amanda Boyce for technical assistance. We thank Susan Banks for her help in preparing the manuscript.

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*Proc. Natl. Acad. Sci. USA*
Vol. 94, pp. 12551–12556, November 1997
Immunology

# Dendritic cell ontogeny: A human dendritic cell lineage of myeloid origin

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*Edited by Irving L. Weissman, Stanford University School of Medicine, Stanford, CA, and approved September 10, 1997 (received for review April 16, 1997)*

ABSTRACT **Dendritic cells (DC) have been thought to represent a family of closely related cells with similar functions and developmental pathways. The best-characterized precursors are the epidermal Langerhans cells, which migrate to lymphoid organs and become activated DC in response to inflammatory stimuli. Here, we demonstrate that a large subset of DC in the T cell-dependent areas of human lymphoid organs are nonactivated cells and belong to a separate lineage that can be identified by high levels of the interleukin 3 receptor α chain (IL-3Rα$^{hi}$). The CD34$^+$IL-3Rα$^{hi}$ DC progenitors are of myeloid origin and are distinct from those that give rise to Langerhans cells *in vitro*. The IL-3Rα$^{hi}$ DC furthermore appear to migrate to lymphoid organs independently of inflammatory stimuli or foreign antigens. Thus, DC are heterogeneous with regard to function and ontogeny.**

Dendritic cells (DC) in lymphoid organs are potent antigen-presenting cells, which play an important role in the initiation of immune responses (1). Studies showing that epidermal Langerhans cells are precursors of DC have suggested that the unique role of DC as "natures adjuvant" is linked to their developmental pathway. Langerhans cells reside in the epidermis where the cells are capable of antigen uptake but have low ability for antigen presentation (2). In response to inflammatory signals, the cells migrate rapidly to lymphoid tissues and differentiate into mature, activated DC with potent ability for stimulation of T cells (3–6). Cells with characteristics of DC precursors have also been found in other tissues (7), and such cells also migrate in response to inflammatory mediators (6, 8). Thus, DC in lymphoid organs have been widely considered to represent the end stage of a stepwise differentiation and migration process, which is completed during inflammation and serves to initiate immune responses (9–12).

Presently, most of the knowledge about the developmental pathway of DC is based on results obtained by cell culture. Cells with characteristics of Langerhans cells and DC can be generated *in vitro* by culture of CD34$^+$ cells in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNF-α) (13–19). Results from studies of *in vitro* colony formation have further indicated that the GM-CSF/TNF-α-responsive progenitors represent a separate DC colony forming cell (15). These and other observations have supported the view that DC are a family of closely related cells that constitute a distinct "DC lineage." However, progenitors committed to become DC have not yet been identified directly in bone marrow. The interpretation of results obtained by colony assays and cell culture is furthermore complicated by the fact that populations of lymphoid progenitors, granulomonocytic progenitors and peripheral blood monocytes also assume characteristics of DC *in vitro* (14, 18–22).

Primitive hematopoietic progenitors and cells committed to become lymphocytes, monocytes, granulocytes, and erythroid cells can be identified as discrete populations of freshly isolated CD34$^+$ bone marrow cells using specific cell surface markers (23–29). Similar characterization of DC progenitors has been difficult due to the lack of selective markers that identify the cells at an early stage of differentiation. In the present study, however, we demonstrate that antibodies to the interleukin 3 receptor α chain (IL-3Rα) selectively react with a large subset of DC in lymphoid organs and identify their precursors in blood and bone marrow. The CD34$^+$IL-3Rα$^{hi}$ progenitors are of myeloid origin but committed to become DC and distinct from those that give rise to Langerhans cells. Unlike Langerhans cells, IL-3Rα$^{hi}$ DC home to lymphoid tissue independently of inflammation or stimulation with foreign antigens.

## MATERIALS AND METHODS

**Tissue.** Tissue from aborted fetuses of gestational age 19–21 weeks was obtained from Advanced Bioscience Resources (Alameda, CA), a nonprofit organization which provides tissue in compliance with state and federal laws. Blood donor buffy coats were obtained from the Stanford Blood Bank (Stanford, CA). Tonsils and adult lymph nodes were obtained from the tissue acquisition service and the clinical flow cytometry laboratory, Department of Pathology, University of Texas Southwestern Medical Center (Dallas).

**Cell Preparation.** Mononuclear cell suspensions were obtained by Lymphoprep gradient centrifugation (Nycomed, Oslo). Antibody-free CD3$^+$CD4$^+$ T cells were isolated to 99% purity from peripheral blood mononuclear cells (PBMC) using CD4 Dynabeads in combination with Detachabead reagent (Dynal, Oslo) after depletion of myeloid cells (CD14$^+$ and CD36$^+$) by Dynabeads (Dynal). Fetal bone marrow CD34$^+$ cells were isolated by positive immunomagnetic selection (Miltenyi Biotech, Auburn, CA), as described (29). Where noted, subsets of immunostained cells were sorted using a FACSVantage flow cytometer (Becton Dickinson).

**Immunophenotypic Analysis.** Multicolor immunofluorescence staining and analysis was performed by standard methods (see ref. 29). Primary or secondary antibodies were conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE),

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Abbreviations: DC, dendritic cell; IL-3Rα, interleukin 3 receptor α chain; GM-CSF, granulocyte/macrophage colony-stimulating factor; G-CSF, granulocyte CSF; M-CSF, macrophage CSF; M-CSFR, M-CSF receptor; TNF-α, tumor necrosis factor α; PBMC, peripheral blood mononuclear cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin; FACS, fluorescence-activated cell sorter.

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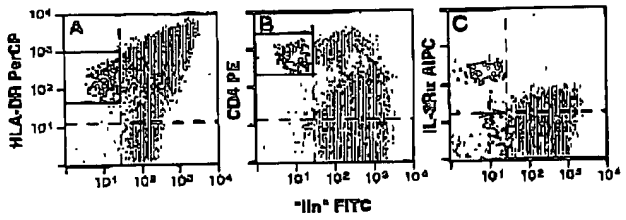


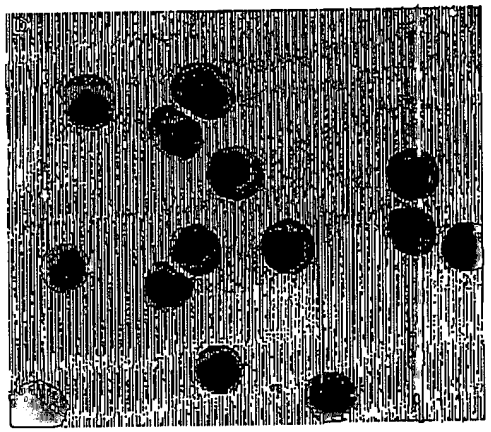

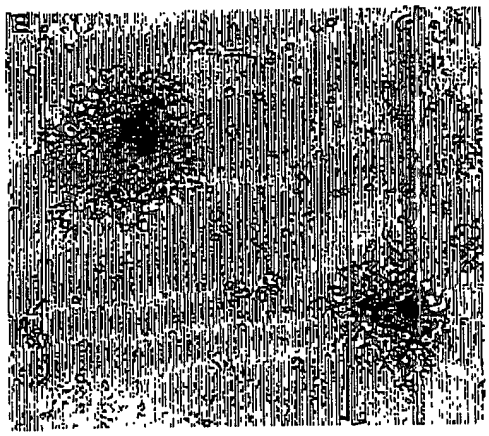

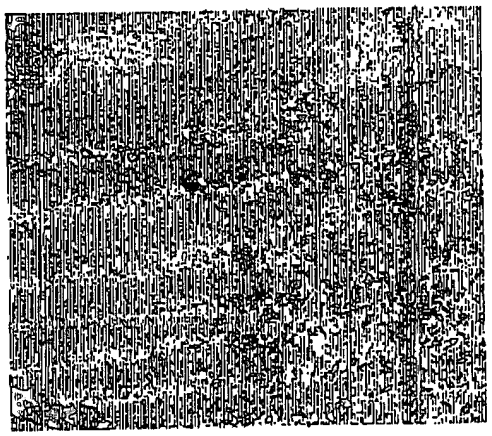

FIG. 1. Antibodies to IL-3Rα selectively stain DC in extrafollicular regions of human tonsils. (*A–C*) Tonsillar mononuclear cells were stained with anti-HLA-DR peridinin-chlorophyll protein (PerCP), CD4 PE, anti-IL-3Rα biotin + Streptavidin allo-phycocyanin (ALPC), and a mixture of FITC-conjugated lineage markers ("lin") for lymphocytes and monocytes (CD3, CD14, CD16, CD19, CD20, CD56, and goat-anti-human IgM). The cells were analyzed by four-color flow cytometry. Dendritic cells were identified as HLA-DR$^+$CD4$^+$lin$^-$—i.e., cells that simultaneously satisfy the criteria of the box regions in *A* and *B*, and are represented by large black dots. Dashed lines represent isotype control levels. (*D*) Wright–Giemsa staining of a cytocentrifuge slide of freshly FACS-sorted tonsillar HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells. (×600.) (*E*) FACS-sorted tonsillar HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells were cultured for 24 h with GM-CSF and IL-3 and photographed *in situ*. (×400.) (*F*) A frozen section of tonsillar tissue was stained with anti-IL-3Rα, biotinylated anti-mouse IgG and streptavidin peroxidase. (×100). Staining was visualized by diaminobenzidine and hydrogen-peroxide, and the section was counterstained with methylene blue.

Table 1. Expression of surface molecules on HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$DC from mononuclear tonsillar cells, PBMC, and fetal lymph node (LN) cells, and on CD34$^+$IL-3Rα$^{hi}$ cells from fetal bone marrow (BM)

| | Tonsil | Fetal LN | PBMC | Fetal BM |
|---|---|---|---|---|
| CD1a | − | − | − | − |
| CD3 | − | − | − | − |
| CD4 | ++/+++ | ++/+++ | ++ | ++ |
| CD5 | − | − | − | − |
| CD11b | − | − | − | − |
| CD11c | − | − | − | − |
| CD13 | −/+ | + | −/+ | + |
| CD14 | − | − | − | − |
| CD15 | − | ND | − | − |
| CD16 | − | − | − | − |
| CD19 | − | − | − | − |
| CD20 | − | − | − | − |
| CD32 | + | + | + | + |
| CD33 | + | +(+) | +(+) | +/++ |
| CD34 | −/+ | + | −/+ | ++/+++ |
| CD36 | ++/+++* | ++/+++ | ++/+++ | ++/++++ |
| CD40 | ++ | + | ++ | + |
| CD45RA | ++(+) | ++(+) | ++(+) | ++ |
| CD45RO | − | − | − | − |
| CD54 | ++/+++ | ND | ++/+++ | +/++ |
| CD56 | − | − | − | − |
| CD58 | + | ND | +/++ | +/++ |
| CD62L | − | + | +/++ | +++ |
| CD64 | − | − | − | − |
| CD80 | − | − | − | − |
| CD86 | + | ND | + | +(+) |
| HLA-DR | ++ | ++ | ++ | ++ |
| HLA-DQ | − | ND | −/+ | − |

Cells were stained as described in legends to Figs. 1, 3, and 4. Mean fluorescence intensity (MFI) levels for the IL-3Rα$^{hi}$ populations are expressed as −, indicating MFI in the first decade on a four log scale, which corresponds to isotype control levels. The +, ++, and +++ indicate MFI in the second, third, and fourth decades, respectively. A/sign means that MFI is on the border between two decades. Parenthesis means that the MFI is in the upper end of a decade. ND, not determined. The data are representative of at least three experiments.

*In one out of three experiments tonsillar HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$DC were negative for CD36.

peridinin-chlorophyll protein, or allo-phycocyanin. Antibodies and streptavidin conjugates were from Becton Dickinson, except CD40, CD86 PE, anti-IL-3Rα biotin, and anti-IL-3Rα PE (PharMingen); CD64 (Meda Rex, West Lebanon, NH); anti-M-CSFR (Santa Cruz Biotechnology); donkey anti-rat IgG PE, goat-anti-mouse IgG PE, and goat-anti-human IgM FITC (Jackson ImmunoResearch). Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

**Media and Cytokines.** Except where specifically noted, cells were cultured in Yssel's medium (30) supplemented with heat-inactivated 10% pooled human AB$^+$ serum and 10% fetal

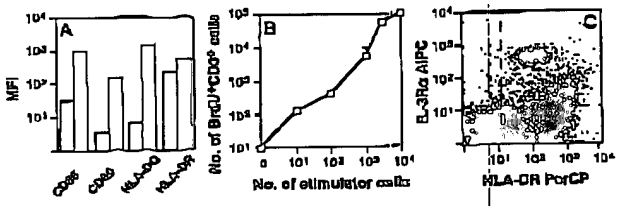


FIG. 2. IL-3Rα$^{hi}$ DC are immature and appear in lymphoid organs independently of stimuli that cause up-regulation of major histocompatibility complex class II and costimulatory molecules. (*A*) Mononuclear cells from tonsil were stained with anti-HLA-DR, anti-IL-3Rα and lineage markers, and either CD86, CD80, HLA-DQ, or isotype control mAbs before (open bars) and after (filled bars) a 16-h incubation at 37°C in Yssel's medium (30). The HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ population was analyzed for mean fluorescence intensity (MFI) staining with the markers indicated on the figure, and the bars represent MFI after isotype control levels were subtracted. (*B*) T cells ($10^5$) were cocultured with indicated numbers of allogeneic IL-3Rα$^{hi}$lin$^-$HLA-DR$^+$ cells from tonsil (stimulator cells). T cell proliferation was measured as the total number of CD3$^+$ BrdU$^+$ cells per well at day 6 of coculture. (*C*) Fetal lymph node cells were stained with anti-HLA-DR, anti-IL-3Rα, and lineage markers (data not shown) and analyzed by flow cytometry as described in Fig. 1*A–C*. Data are representative of three experiments.

bovine serum. Recombinant human cytokines were used as noted at the following concentrations: GM-CSF (10 ng/ml), IL-3 (10 ng/ml), IL-6 (500 units/ml), IL-7 (50 ng/ml) (all from Collaborative Biomedical Products, Bedford, MA), erythropoietin (2.5 units/ml; CILAG, Schaffhausen, Switzerland), stem cell factor (40 ng/ml; Peprotech, Rocky Hill, NJ), granulocyte-CSF (G-CSF, 50 ng/ml, Amgen Biologicals), and macrophage-CSF (M-CSF, 10 ng/ml; R & D Systems).

**Cell Cultures.** IL-3Rα$^{hi}$lin$^-$HLA-DR$^+$ cells from tonsil and PBMC and CD14$^{hi}$ monocytes from PBMC were sorted by fluorescence-activated cell sorter (FACS) and cultured for 36 h before mixing with T cells to allow maturation of DC precursors. GM-CSF and IL-3 were added to IL-3Rα$^{hi}$lin$^-$HLA-DR$^+$ cells to enhance survival. Stimulator cells were washed twice before coculture with CD4$^+$ T cells ($10^5$ per well) in flat-bottomed 96-well plates for 6 days. Bromodeoxyuridine (BrdU; Sigma) (50 μM) was added 12 h before harvest latex particles ($10^5$ per well) were added immediately prior to harvest as a reference for cell counts (29). The cells were stained with CD3 PE, followed by fixation, permeabilization, staining with anti-BrdU FITC, and flow cytometric analysis, as described (29). Cocultures with stimulator cells derived by culture of fetal bone marrow cells were pulsed with 1 μCi (1 Ci = 37 GBq) [$^3$H]thymidine for 8 h before collecting and counting.

## RESULTS AND DISCUSSION

**IL-3Rα Is a Selective Marker for a Large Subset of DC in T Cell-Rich Zones of Human Peripheral Lymphoid Organs.** Human tonsils contain DC that can be identified as cells that lack lineage markers for monocytes and lymphocytes (lin$^-$) and are positive for HLA-DR and CD4 (HLA-DR$^+$CD4$^+$lin$^-$) (Fig. 1*A* and *B*) (31, 32). In an attempt to identify DC-selective markers, we screened antibodies to leukocyte differentiation antigens for selective reactivity with this population. Antibodies to IL-3Rα reacted strongly with more than 85% of HLA-DR$^+$CD4$^+$lin$^-$ cells (0.32–0.37% of tonsillar mononuclear cells, $n = 3$), but weakly with most other cells ($n = 3$) (Fig. 1*C*). The staining was sufficiently specific to allow a 200-fold enrichment of HLA-DR$^+$CD4$^+$lin$^-$ cells with a single positive immuno-magnetic selection ($n = 2$). HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells were also found in adult cervical, axillar, intramammary, mesenteric, and femoral lymph nodes. The frequency ranged from 0.1%–1.8% (average 0.5%, $n = 9$). In all cases, cells staining brightly with the anti-IL-3Rα were found within the HLA-DR$^+$lin$^-$ population and constituted the majority of these cells (on average 60.2 ± 11.2%, $n = 9$).

Freshly sorted HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells showed an immature morphology without cytoplasmic protrusions (Fig. 1*D*). The majority of the cells died rapidly in culture, but could be partially rescued by the addition of cytokines (GM-CSF and IL-3). Under these conditions, the cells rapidly formed large aggregates of cells as previously reported for tonsillar DC (32) (Fig. 1*E*). After 3–5 days the cells were more dispersed and showed multiple long processes characteristic of DC (data not shown).

IL-3Rα$^{hi}$ cells were found almost exclusively in the T cell-rich extra-follicular regions of the tonsil (Fig. 1*F*). The localization as well as the the cytokine requirements and phenotype of the HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells (Table 1) suggest that they are identical to the "plasmacytoid T cell" DC that were recently characterized by Grouard *et al.* (33).

**IL-3Rα$^{hi}$ DC Are Immature and Appear in Lymphoid Organs Independently of Stimuli That Cause Up-Regulation of Major Histocompatibility Complex (MHC) Class II and Costimulatory Molecules.** Originally, it was concluded that DC in lymphoid tissues such as tonsils are activated, mature antigen-presenting cells, because isolated cells expressed high levels of MHC class II and costimulatory molecules (31, 32). However, in those studies the cells were isolated after 1–2 days of cell culture. As shown in Fig. 2*A* and Table 1, HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells in fresh preparations of tonsillar mononuclear cells expressed low levels of CD80 (B7.1), CD86 (B7.2), and HLA-DQ. Overnight culture of unseparated cells in the absence of cytokines was sufficient to induce the mature phenotype (Fig. 2*A*). Sorted HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells that were allowed to mature in culture for 36 h and kept viable with GM-CSF and IL-3 were potent stimulators of allogeneic CD4$^+$ T cells (Fig. 2*B*). These results are surprising in view of the hypothesis that DC migrate to lymphoid organs in response to signals that lead to cell activation. However, these data are in agreement with those of Grouard *et al.* (33), who also studied freshly isolated cells, and with previous reports

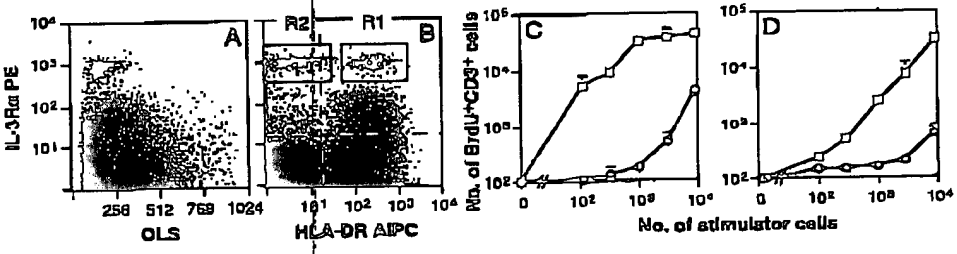


FIG. 3. HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ DC are present in peripheral blood. (*A* and *B*) PBMC were stained with anti-HLA-DR, anti-IL-3Rα, and lineage markers (not shown) and analyzed by flow cytometry as described in Fig. 1*A–C*. R1 and R2 in *B* represent regions used to sort IL-3Rα$^{hi}$ blood cells that were positive or negative for HLA-DR, respectively. (*C* and *D*) HLA-DR$^+$lin$^-$IL-3Rα$^{hi}$ cells sorted from blood according to R1 in *B* were first cultured separately with IL-3 and GM-CSF for 36 h and then incubated with allogeneic (*C*) or autologous (*D*) CD4$^+$ T cells. T cell proliferation was measured as total number of CD3$^+$BrdU$^+$ cells per well at day 6 of coculture with indicated numbers of IL-3Rα$^{hi}$lin$^-$HLA-DR$^+$ cells (□) or CD14$^{hi}$ monocytes (○) from the same donor (stimulator cells). Individual displays show data that are representative of three experiments. OLS, ortogonal light scatter.

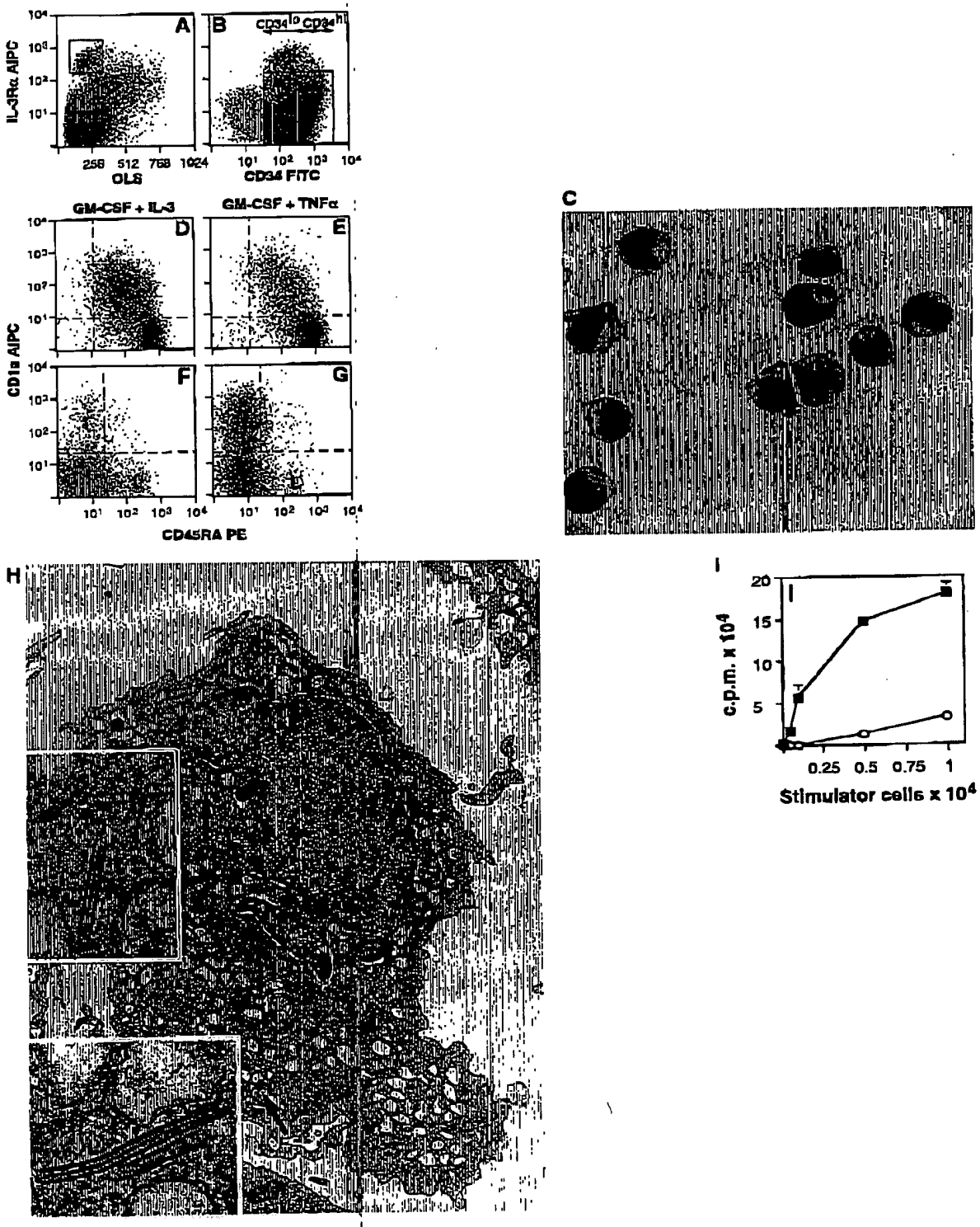


FIG. 4. Proliferating progenitors for IL-3Rα$^{hi}$ DC are found as a discrete CD34$^+$IL-3Rα$^{hi}$ population that is distinct from the cells that give rise to Langerhans cells in response to GM-CSF and TNF-α. (*A* and *B*) Isolated CD34$^+$ fetal bone marrow cells were stained with CD34 and anti-IL-3Rα. The cells were analyzed by flow cytometry as described in Fig. 1 *A–C*. The CD34$^+$IL-3Rα$^{hi}$ population was defined according to the region in *A* (blue dots). CD34$^+$IL-3Rα$^{lo}$ cells were defined according to the region in *B* (red dots). OLS, orthogonal light scatter. (*C*) Freshly sorted Wright–Giemsa-stained CD34$^+$IL-3Rα$^{hi}$ cells display mitotic figures. (×600.) (*D–G*) CD34$^+$IL-3Rα$^{hi}$ cells (blue) and CD34$^+$IL-3Rα$^{lo}$ cells (red) were sorted according to the regions in *A* and *B*, respectively, and cultured in the presence of indicated cytokines, stained with CD1a and CD45RA after 5 days of culture, and analyzed by FACS. L = B lymphoid cells staining brightly with CD19 (data not shown). None of the cultured CD34$^+$IL-3Rα$^{hi}$ cells (*D* and *E*) stained positively with CD19 (data not shown). Dashed lines indicate isotype control levels. (*H*) Transmission electron microscograph (×5,000) of a CD1a$^+$ cell sorted from CD34$^+$IL-3Rα$^{lo}$ cells cultured with GM-CSF and TNF-α, as described in *G*. The arrows point to areas containing Birbeck granules, (shown in *Insets*, ×45,000). (*I*) CD4$^+$ T cells ($10^5$) were cocultured with indicated numbers of

showing that DC in murine spleens were immature immediately after isolation and differentiated rapidly *in vitro* (34, 35).

The nonactivated phenotype of DC in tonsils may seem like a paradox since tonsils are typically removed after repeated inflammations. One can further not exclude the possibility that the isolated cells had migrated to lymphoid tissue in response to inflammation, but not yet assumed the activated phenotype. To determine whether the presence of HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ cells in lymphoid tissue depends on previous exposure to foreign antigens or inflammatory stimuli, we examined whether the cells could be found in fetal lymph nodes. Presumably, these lymph nodes drain sterile, noninflamed tissues. As shown in Fig. 2*C*, HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ cells were present at high frequencies in the fetal lymph nodes (2.6 ± 1.1%, $n = 3$). These cells were more frequent than cells expressing high levels of myeloid markers, such as CD13 and CD33 (1.5 ± 0.2%, $n = 3$). The HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ cells in fetal lymph nodes expressed the same combination of markers as those in tonsils (Table 1). This cell type is therefore most likely capable of migrating to lymphoid tissue independently of inflammation and foreign antigens and without initiating immune responses.

**IL-3R$\alpha^{hi}$ DC Are Present in Peripheral Blood.** HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ cells were readily detectable as a population with low orthogonal light scatter in blood from adult donors, and constituted 0.47 ± 0.14% ($n = 8$) of PBMC (Fig. 3 *A* and *B*, R1). A second population of IL-3R$\alpha^{hi}$ cells was present among PBMC, but these cells were HLA-DR$^-$ (Fig. 3*B*, R2) and were found to be basophilic granulocytes (data not shown). The HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ blood cells expressed the same combination of markers and had similar morphology as shown for the tonsillar counterparts (Table 1 and data not shown). However, an interesting difference was that whereas tonsillar DC did not express the lymph node homing molecule L-selectin (CD62L), the cells from PBMC were positive for this marker (Table 1).

Whereas multiple cell types can stimulate allogeneic T cells, DC are characterized by their higher potency relative to other antigen-presenting cells (13, 15, 21, 31, 33, 36–39). The cells also induce proliferation of autologous T cells *in vitro* (37). The data in Fig. 3 *C* and *D* demonstrate that HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ blood cells were up to 100-fold more potent than monocytes in stimulating both allogeneic and autologous T cells. Cells with similar characteristics have previously been identified in blood and were referred to as CD11c$^-$ DC or CD33$^{dim}$CD14$^-$CD16$^-$ DC (36, 37).

**Precursors of IL-3R$\alpha^{hi}$ DC Are Found Among CD34$^+$ Bone Marrow Cells and Are Distinct from the Cells That Give Rise to Langerhans Cells in Response to GM-CSF and TNF-$\alpha$.** IL-3R$\alpha^{hi}$ cells were readily identified as a distinct subset of CD34$^+$ fetal bone marrow cells with low orthogonal light scatter (3.1 ± 0.9%, $n = 5$) (Fig. 4 *A* and *B*). The majority of the CD34$^+$IL-3R$\alpha^{hi}$ cells were within the CD34$^{lo}$ subset, which contains lineage-committed progenitors (Fig. 4*B*) (23). The CD34$^+$IL-3R$\alpha^{hi}$ cells had morphology and immunophenotype similar to IL-3R$\alpha^{hi}$lin$^-$HLA-DR$^+$ cells in blood and tonsil, but appeared more immature, and mitotic figures were frequently observed (Fig. 4*C* and Table 1).

Previous studies have demonstrated that DC and Langerhans cells can be derived by culture of CD34$^+$ cells with GM-CSF and TNF-$\alpha$ (13–19). The subset of CD34$^+$ cells that contains these TNF-$\alpha$-dependent progenitors has not yet been identified. We therefore examined whether they were identical to the CD34$^+$IL-3R$\alpha^{hi}$ cells. The sorted IL-3R$\alpha^{hi}$ population (Fig. 4*A*) formed aggregates of cells with DC morphology during culture with either GM-CSF and IL-3 or GM-CSF and TNF-$\alpha$ (data not shown), and after 5 days the cells were positive for CD1a (Fig. 4 *D* and *E*). However, differentiation into DC occurred independently of TNF-$\alpha$, and the CD1a$^+$ cells co-expressed CD45RA, which is absent from Langerhans cells (39) (Fig. 4 *D* and *E*). Tonsillar HLA-DR$^+$lin$^-$IL-3R$\alpha^{hi}$ cells also obtained a CD1a$^+$CD45RA$^+$ phenotype when cultured under the same conditions (data not shown). In contrast, sorted CD34$^+$ cells with low levels of the IL-3R$\alpha$ (Fig. 4*B*) gave rise to few CD1a$^+$ cells when cultured with GM-CSF and IL-3, but large numbers of CD45RA$^-$CD1a$^+$ cells in the presence of GM-CSF and TNF-$\alpha$ (Fig. 4 *F* and *G*). Consistent with a phenotype of Langerhans cells, 30–40% of these CD45RA$^-$CD1a$^+$ cells contained Birbeck granules (Fig. 4*H*). The frequency of Birbeck granule-positive cells among CD1a$^+$ cultured CD34$^+$IL-3R$\alpha^{hi}$ cells was less than 4%, and may therefore reflect contamination of sorting gates. Finally, CD34$^+$IL-3R$\alpha^{hi}$ cells cultured with GM-CSF and IL-3 were potent stimulators of allogeneic CD4$^+$ T cells, and more potent than CD14$^+$ macrophages generated by culture of CD34$^+$M-CSFR$^+$ cells from the same donor in M-CSF (Fig. 4*I*).

**IL-3R$\alpha^{hi}$ DC Are of Myeloid Origin.** The distribution of M-CSF receptor (M-CSFR) and IL-3R$\alpha$ among CD34$^+$ cells suggested that CD34$^+$IL-3R$\alpha^{hi}$ cells may derive from cells in the M-CSFR$^{hi}$ population, which down-regulate the M-CSFR as they up-regulate the IL-3R$\alpha$ (arrow in Fig. 5*A*). Because M-CSFR expression on CD34$^+$ cells is restricted to granulomonocytic progenitors, this would indicate that IL-3R$\alpha^{hi}$ cells belong to the granulomonocytic lineage (29). To test this possibility, immature progenitors (i.e., CD34$^{hi}$, see Fig. 4*B*) with high levels of M-CSFR and low levels of IL-3R$\alpha$ (region in Fig. 5*A*) were sorted and cultured. After 60 h of culture two populations of cells that had downmodulated the M-CSFR were observed, with high and low levels of IL-3R$\alpha$, respectively (Fig. 5*B*). These cells were sorted and cultured for 5 additional days in medium supplemented with GM-CSF and IL-3. At this stage, the cultures from the IL-3R$\alpha^{lo}$ population contained CD15$^+$ granulocytic cells (Fig. 5*C*) whereas cultures from the IL-3R$\alpha^{hi}$ population contained CD1a$^+$ cells with DC morphology (Fig. 5*D* and data not shown). The latter cells induced strong proliferation of allogeneic T cells compared with equal numbers of CD14$^+$ macrophages generated by culture of CD34$^+$M-CSFR$^+$ cells for 5 days with M-CSF (29) ($n = 2$, data not shown). Addition of TNF-$\alpha$ to the medium did not increase the number of CD34$^+$IL-3R$\alpha^{hi}$ cells generated from M-CSFR$^{hi}$ myeloid progenitors, further suggesting that the cells are distinct from the TNF-$\alpha$-dependent Langerhans cell progenitors (data not shown).

**Thymus Contains Small Numbers of IL-3R$\alpha^{hi}$ DC That Express Low Levels of CD34.** Earlier studies have demonstrated that some DC may share the differentiation pathway of the T cell lineage and that DC are generated by progenitors in the thymus (18, 19, 40). We therefore investigated whether IL-3R$\alpha^{hi}$ DC were present in the thymus. The results showed that less than 0.1% of the total thymic cell population were IL-3R$\alpha^{hi}$ DC and that the IL-3R$\alpha^{hi}$ cells constituted only 15 ± 2% ($n = 3$) of HLA-DR$^+$lin$^-$ subset. This may indicate that other DC populations than the IL-3R$\alpha^{hi}$ cells are predominant in thymus. In addition, the IL-3R$\alpha^{hi}$ DC in thymus expressed low levels of CD34 compared with bone marrow IL-3R$\alpha^{hi}$ cells from the same donor (data not shown). Similar low levels of CD34 were found on the cells in fetal lymph nodes (Table 1). It therefore seems likely that the few IL-3R$\alpha^{hi}$ DC in the thymus represent cells that have migrated from bone marrow and are distinct from thymic DC progenitors.

**IL-3R$\alpha^{hi}$ DC Constitute a Separate Lineage of DC That Follow a Differentiation Pathway Distinct from Langerhans Cells.** The data presented here demonstrate that a subset of DC in human

allogeneic DC (□) or macrophages (○) from the same donor. The DC were generated by culturing sorted CD34$^+$IL-3R$\alpha^{hi}$ cells for 5 days with GM-CSF and IL-3 (□). The macrophages were generated by culture of CD34$^+$M-CSFR$^+$ cells for 5 days with M-CSF and purified by FACS sorting of CD14$^+$ cells. T cell proliferation was measured as incorporation of [$^3$H]thymidine at day 6 of coculture. c.p.m; counts per minute. Data are representative of three experiments.

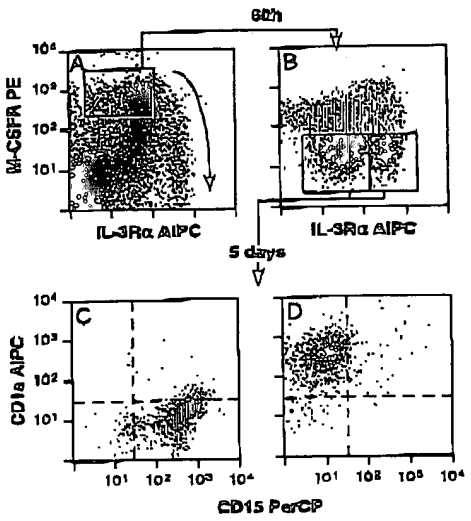


FIG. 5. IL-3Rα$^{hi}$ DC follow the myeloid differentiation pathway to the branching point of the granulocytic and monocytic lineages. (*A*) CD34$^+$ cells were incubated for 12 h in serum-free medium (25) to allow up-regulation of the M-CSFR and stained with CD34, anti-M-CSFR, and anti-IL-3Rα. The arrow shows the suggested differentiation pathway of CD34$^+$IL-3Rα$^{hi}$ cells. The region shows sorting criteria for M-CSFR$^{hi}$/IL-3Rα$^{lo}$ cells. An additional gate was set to include only CD34$^{hi}$ cells, shown in Fig. 4*B*, to restrict the sort to immature progenitors (29). (*B*) After a 60-h culture of CD34$^{hi}$M-CSFR$^+$ cells in serum-free medium (25) containing stem cell factor, G-CSF, GM-CSF, IL-3, and IL-6, the cells were stained with anti-M-CSFR and anti-IL-3Rα. The regions indicate criteria for sorting of the two populations that had downmodulated the M-CSFR during the culture period. (*C* and *D*) After a 5-day secondary culture of IL-3Rα$^{lo}$M-CSFR$^{lo}$ cells (green) and IL-3Rα$^{hi}$M-CSFR$^{lo}$ cells (blue) with IL-3 and GM-CSF, the cells were stained with CD1a and CD15 and analyzed by FACS. The small subset of CD15$^{lo}$ cells in *C* represent basophilic granulocytes (27, 29).

lymphoid organs and blood represent a separate lineage of cells. The cells can be readily identified and isolated on the basis of their high levels of the IL-3Rα. Their precursors in the bone marrow appear to follow the myeloid differentiation pathway to the branching point of the granulocytic and monocytic lineages. Progenitors that have committed to this DC lineage are distinct from those that give rise to Langerhans cells when cultured in the presence of GM-CSF and TNF-α. Their progeny furthermore lack several characteristics of Langerhans cells that were previously considered common to DC. Unlike Langerhans cells, the IL-3Rα$^{hi}$ DC appear to undergo little differentiation during transit from the bone marrow to lymphoid organs. The IL-3Rα$^{hi}$ DC further seem capable of migrating to lymphoid tissue independently of inflammatory stimuli. The results concur with previous reports showing that some DC spend less than 24 h in transit from the progenitor pool to lymphoid tissue and that DC turnover occurs constantly during steady state conditions (1, 41–43). Thus, the DC system is constituted by multiple cell types with distinct developmental pathways and functional properties. A large proportion of the cells may enter lymphoid tissue without inducing immune responses.

We thank Drs. Lewis Lanier, Anne O'Garra, Rene de Waal Malefyt, and Gosse Adema for critical reading of the manuscript; David Houck for expert assistance with cell sorting; and Barny Abrams, Dr. Qudrat Nasraty, and Dr. Kenneth A. Davis for conjugation of antibodies. J.O. and F.L.-J. were supported by grants from the Norwegian Research Counsel. DNAX Research Institute is supported by Schering-Plough.

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